Potentials and pitfalls of epigenetic editing in gene-specific re-expression of epigenetically silenced genes

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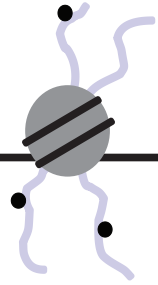

General introduction

EPIGENETICS

"An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (1). With this definition, a field of research is described that received greatly increasing attention in the last decade (2, 3). Because of the increase of efforts in the field of epigenetics, a wealth of information has become available. Primarily, changes in epigenetic marks are associated with a change in chromatin condensation, altering the accessibility of genes for transcription factors. In this way, levels of gene expression are regulated, which is crucial for development of organisms and normal functioning of cells. Several processes fall under the term 'epigenetics': whereas more and more is discovered about the functions of non-protein coding RNAs (4), nucleosome positioning and density (5) histone variants (6) and prions (7) in epigenetic regulation of gene expression, the most intensively investigated epigenetic mechanisms are DNA methylation and histone modifications.

DNA methylation

In mammalian cells, cytosines, mainly when preceding guanines (CpGs), can become methylated at their C5 position through the action of DNA methyltransferases (Dnmt1, Dnmt3a and Dnmt3b) (8). Whereas Dnmt3a and Dnmt3b are known for executing *de novo* DNA methylation, Dnmt1 is taking care of maintenance of DNA methylation upon cell division. CpGs, the main target for DNA methylation in mammalian DNA are often present in clusters, called islands. More than half of the genes in vertebrates contain such CpG islands (9). If cytosines in CpG islands close to the transcription start site in promoters of genes are methylated, of which most is known, this is associated with gene silencing through several possible pathways (10). CpG methylation can hamper activating transcription factors from binding, the methylated CpGs can be bound by methyl binding proteins which recruit co-repressors, or the DNMTs recruit histone modifying enzymes, all leading to repression of gene expression.

Importantly, this DNA methylation is reversible, which might be necessary to activate gene expression. Whereas DNA demethylation can occur passively by inhibiting the maintenance DNA methyltransferase subsequent to cell division, there is also evidence for active DNA demethylation, as reviewed in (11). Until recently it was not generally accepted that active DNA demethylation occurs in mammals, even though examples have been described of both global and locus specific active DNA demethylation (11). For example, the interleukin-2 promoter-enhancer region is demethylated within twenty minutes upon stimulation in T lymphocytes. In this short time frame, passive DNA demethylation could not have occurred. In another example, the promoter of the brain-derived neurotrophic factor is demethylated in post-mitotic cells. Since post-mitotic cells do not divide, passive DNA demethylation can not be the cause (11).

Although these evidences on active DNA demethylation caused a general acceptance of the phenomenon, the exact mechanism remains elusive. In the past years several potential mechanisms and associated candidate proteins have been suggested, as extensively reviewed (11, 12, 13, 14, 15, 16, 17). Currently, the most acknowledged possible pathway of active DNA demethylation is through oxidation of 5-methylcytosine to 5-hydroxymethylcytosine by Tet proteins (18, 19).

Histone modifications

The human DNA, consisting of around 3 billion nucleotides comprising 20,000-25,000 protein encoding genes, covers a length of about 2-3 meters in uncondensed state. Thus, for all of this to fit into the nucleus of cells in an organized manner, 147 bp of DNA is wrapped around histone cores. 147 bp of DNA, together with the histone core and the interconnecting linker DNA is known as a nucleosome. The density of such nucleosomes thus regulates chromatin condensation, also important for gene expression regulation. The histone core octamer of nucleosomes consists of two dimers of histone H2A and H2B, binding a tetramer consisting of two copies each of histone H3 and H4. Importantly, each histone monomer has a protruding N-terminal tail which can be posttranslationally modified. Several types of such modifications have been observed, including methylation, acetylation, phosphorylation and ubiquitination (20). Depending on the exact amino acid residue modified but also on the quantity of modification marks (up to three methyl groups can be added) and the type of modification, such posttranslational modifications have been associated with repressed or activated genes (21). A nomenclature has been created for describing this list of histone modifications. First the histone of which the tail is modified is named, then the amino acid residue that is modified, followed by the modification and the amount of this posttranslational modification that is present (22). For example H3K27me3 represents trimethylation of lysine 27 of histone 3. Variants of the histones increase the complexity of gene expression regulation even more, executing a variety of influences on gene expression (6).

For almost every specific modification there is an enzyme in charge (or more than one). Moreover, even for the specific addition or removal of the first, second or third methyl group on one histone tail residue different enzymes can be needed. This list of enzymes responsible for inducing (writers) or removing (erasers) posttranslational histone tail modifications is still extending (23).

Furthermore, there are 'readers' that bind to specific histone modifications (24). It is suggested that these 'reader' proteins recognize specific histone modifications through e.g. their bromodomains or chromodomains and subsequently influence gene expression by their effector domain. There are also reader proteins that do not have enzymatic

activity but merely recruit enzymes to execute the effect. This suggests the presence of a certain histone code, indicating that (combinations of) histone modifications determine the state of gene expression (25, 26). However, further investigation is necessary to determine whether epigenetic marks are indeed instructive for gene expression or if they are merely a consequence (27, 28).

EPIGENETICS IN DISEASE

In the normal situation, epigenetic regulation is especially important in development, establishing diversity in cellular phenotypes of an organism despite the fact that each cell type has the same genotype (29, 30). In later stages of development, the inheritability of epigenetic marks ensures maintenance of the gene expression regulation and thereby the phenotype of cells after cell division (31). Interestingly, there is also evidence for chromatin to escape the resetting of the epigenetic state in the germline, causing transgenerational inheritance (32, 33, 34). This transgenerational epigenetic inheritance seems less likely to occur at a broad scale but is probably important for e.g. the repression of retrotransposons (33). Recent indications imply that transgenerational inheritance of gene expression regulation is likely not so much caused by DNA methylation and histone modifications as it is by RNA-mediated pathways (33).

However, whether received from a parent or induced by environmental factors, errors can occur in epigenetic mechanisms, causing unwanted changes in epigenetic marks. Ultimately, such epimutations can lead to aberrant gene expression silencing or activation. In addition to the long known genetic mutations, many diseases have now been associated with epimutations (35, 36). The research into the role of epigenetics in diseases has mainly focused on cancer, where e.g. aberrant silencing of tumor suppressor genes causes uncontrolled cell growth (3, 37, 38). Interestingly, as it is now known that epigenetic modifications play a role in diseases like cancer, the epigenetic marks are currently used and developed as diagnostic or prognostic markers (39, 40, 41). Importantly, epigenetic marks are reversible (42), which provides potential for therapeutic intervention. Indeed, various epigenetic therapies have been developed to inhibit enzymes involved in DNA methylation and histone modifications (42, 43). Thus far, mainly inhibitors of DNA methyltransferases and histone deacetylases are in use, but inhibitors of other epigenetic enzymes are in development as well (43). Some of the DNMT and HDAC inhibitors have been FDA approved and are currently used in the clinic for treatment of hematological malignancies (42, 43). Recently, progress has been achieved in treatment of solid tumors with epigenetic drugs (44).

Despite these successes, risks and downsides of these drugs have been suggested as well (45, 46, 47). The major risk of epigenetic drugs is that inhibition of the epigenetic enzymes could lead to genome-wide consequences, causing unwanted side-effects by

inducing expression of genes that should remain silenced (Fig. 1a) (48). In addition, histone modifiers often affect non-histone proteins as well, so upon inhibition of these histone modifying enzymes, other processes are affected too (49, 50). Another disadvantage is that the currently FDA-approved epigenetic drugs are designed to upregulate the expression by acting against silencing epigenetic processes and will thus not be applicable to downregulate aberrantly expressed genes. Furthermore, whereas the inhibition of DNA methylation by epigenetic drugs is beneficial for upregulation of, for example, expression of a tumor suppressor gene, the stability of the genome is endangered. To be exact, genome-wide DNA hypomethylation causes DNA recombination, resulting in instability of the genome (51). Moreover, the FDA-approved drugs inhibit epigenetic enzymes and upon clearance of the drug the unaffected endogenous epigenetic marks will likely recruit the recovered target enzyme. Consequently, the initial prevention of the epigenetic mark will be reset and/or remaining marks could recruit repressive machinery.

The temporary nature of the effect of current epigenetic drugs also applies to gene-specific approaches like siRNA (degradation of mRNA) and cDNA (introduction of the gene of interest). siRNA has to be delivered repeatedly because of the constant production of mRNA. Introduction of cDNA into cells only results in expression of this one isoform of the gene of interest, causing problems in gene function (52). In addition, depending on the promoter used, the gene is expressed in abnormal concentrations. Another approach for gene-specific gene expression modulation is Epigenetic Editing.

EPIGENETIC EDITING

In this thesis, the potentials of Epigenetic Editing were investigated. Epigenetic Editing is the gene-specific rewriting of epigenetic marks in order to modulate expression of endogenous genes (53). This approach has the potential to adverse all of the disadvantages of the current epigenetic drugs as mentioned above (Fig. 1b). The principal concept of Epigenetic Editing is the fusion of a gene specific DNA binding domain to an epigenetic effector domain, thereby forcing presence of the epigenetic effector domain on the transcription start site of the gene of interest (Fig. 2). The targeted writer or eraser of epigenetic marks can subsequently rewrite the epigenetic marks, ultimately leading to gene expression modulation.

The main advantage, obviously, is the gene-specificity of the approach (Fig. 1b), caused by engineering DNA binding domains that recognize specifically the gene of interest. Using such DNA binding domains enables the targeting of virtually any (currently undruggable) gene. Furthermore, the (combinations of) epigenetic effector domains will cause a change in epigenetic marks, which can be inherited upon cell division, ensuring a sustained effect. Depending on the epigenetic effector domain

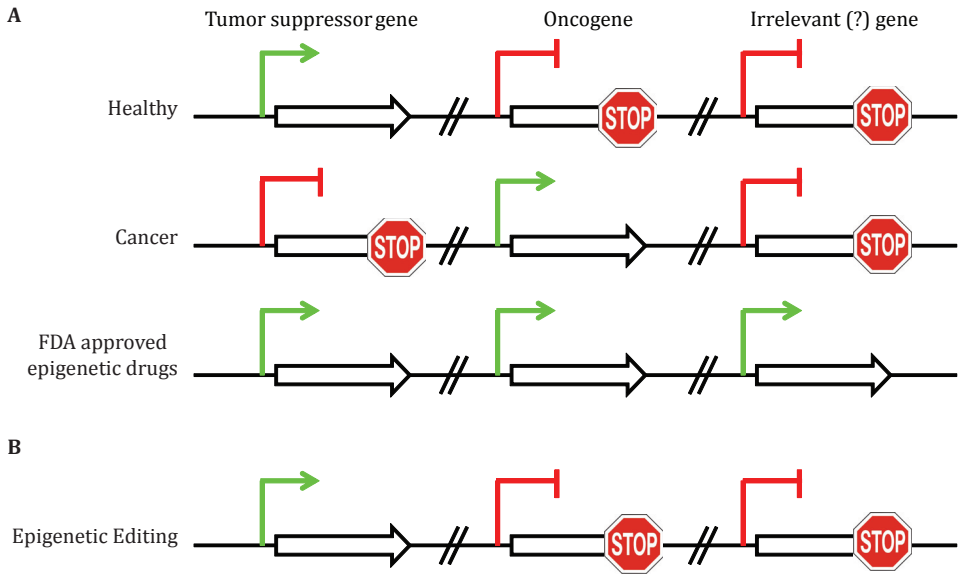

Figure 1. Epigenetic editing is gene-specific
In (A), gene-expression status of tumor suppressor genes, oncogenes and 'irrelevant' other genes is schematically represented in the healthy situation, in cancer and upon treating cancer cells with (genome-wide acting) epigenetic drugs. In (B), the major advantage of Epigenetic Editing, its gene-specificity, is represented.

fused to the DNA binding domain, both up- and downregulation can be envisioned. Importantly, the epigenetic interference will lead to expression or repression of all isoforms of a gene and expression will be regulated by the natural gene expression regulation mechanisms. Another advantage is the targeting of only two alleles of a gene, whereas e.g. siRNA needs to act against big amounts of constantly produced RNA. In addition to the advantages for using Epigenetic Editing as a therapeutic approach, Epigenetic Editing is of interest for investigating fundamental epigenetic questions, for example related to cause and consequence of epigenetic marks with respect to gene expression (27, 28).

DNA binding domain

For gene-specificity, engineerable DNA binding domains like zinc fingers, triple helix forming oligonucleotides (54) and TALe domains (55) can be used. In this thesis, zinc fingers were used, which are found in many natural transcription factors and can be engineered to recognize any target sequence of interest (56). One zinc finger recognizes three to four base pairs of DNA and a table has been created to identify the zinc finger needed to target the triplet of interest (56). Using these modular building blocks (57), multiple zinc fingers can be stitched together. When stitching six zinc fingers together,

recognizing 18 base pairs, from a mathematical point of view a unique address in the human genome can be targeted (58). In this way, also the zinc fingers recognizing the EpCAM promoter, which were used in this thesis, have been engineered (59).

Engineering and testing of zinc fingers can also be performed using a zinc finger library (60), combined with further optimization (61). The library consists of zinc finger proteins fused to a gene expression modulating domain. Thus, the binding zinc finger can be traced back by screening for an effect on gene expression of the gene of interest. The ICAM-1 zinc finger used in this thesis has been selected in this way and further optimized (61).

Much progress has been achieved so far using engineered zinc fingers. In fusions to transient transcriptional activation domains or nucleases (62) (for gene correction purposes) zinc fingers have entered clinical trials. Moreover, in fusion to certain epigenetic enzymes (Epigenetic Editing), endogenous genes have been silenced (63, 64, 65).

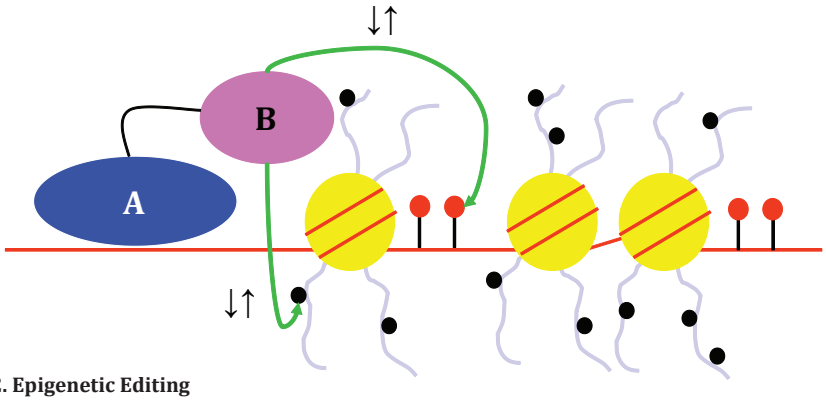

Figure 2. Epigenetic Editing

Here, the general concept of Epigenetic Editing is schematically shown. **A** represents the gene-specific DNA binding domain, **B** represents the epigenetic effector domain. Arrows show the writing or erasing of epigenetic marks by the fusion protein. (see **chapter 2** for more detailed explanation)

Epigenetic effector domains

Depending on the epigenetic marks at the gene of interest and the desired effect, many epigenetic enzymes serve as interesting candidates for the modulation of target gene expression by fusion to zinc fingers. To repress gene expression, enzymes that methylate DNA or induce repressive histone modifications like H3K9me3 or H3K27me3 are of interest to fuse to the DNA binding domain, whereas enzymes removing H3K4me3 or histone acetylation (associated with active genes) could also lead to repression of gene expression. To re-activate epigenetically silenced genes, enzymes that perform the reverse process can be fused to DNA binding domains. Thus, DNA demethylases or e.g. an H3K27 demethylase or a histone acetyltransferase are of interest to fuse to DNA binding domains for re-activation of gene expression.

AIM AND OUTLINE OF THIS THESIS

The overall aim of this thesis is to identify potent writers/erasers of epigenetic marks to gene-specifically induce expression of epigenetically silenced genes by rewriting their epigenetic signatures. For achieving this aim, two questions need to be answered; (1) does targeting of the candidate (domain of the) epigenetic enzyme result in gene-specific targeted removal of repressive epigenetic marks or induction of epigenetic marks associated with active genes and (2) does this subsequently result in achievement of re-activation of gene expression.

In **chapter 2**, previously reported efforts on targeting epigenetic enzymes to predetermined sites are reviewed. As described in **chapter 2**, successful induction and removal of epigenetic marks has been achieved by sequence-specific targeting of various epigenetic enzymes. This sequence-specific targeting is targeting by fusion to a sequence-specific DNA binding domain which is not gene-specific. In addition, several of such targeting studies show activation or repression of target gene expression. Importantly, few studies report efficient gene-specific repression of endogenous genes through Epigenetic Editing (63, 64, 65) However, no reports on Epigenetic Editing to gene-specifically induce gene expression have been published as far as known. In addition to the description of effects on epigenetic and gene expression level, in **chapter 2** factors are distilled from the reported studies that are likely to be of influence on the efficacy of Epigenetic Editing.

To identify potent enzymes to achieve re-activation of epigenetically silenced genes, several experimental set-ups have been used to screen epigenetic enzymes (mainly putative DNA demethylases) for their effect on epigenetic marks and gene expression in **chapter 3**. In addition to analyzing genome wide effects upon overexpressing the epigenetic enzymes and the use of cotransfection studies to assess untargeted and targeted effects of the enzymes, cell systems were used with large integrations of repeats of DNA binding domain recognition sites. This large amount of LacO site repeats, to which the LacR DNA binding domain can bind, is an interesting system to screen for the effect of targeted epigenetic enzymes. First of all, the large repeat of LacO sites ensures the presence of a large amount of LacR-targeted epigenetic enzyme in the same region. Moreover, the repeats are integrated within the genome of mammalian cells, more closely resembling the natural situation of chromatin in a repressed state than plasmids.

Towards gene-specific and endogenous targeted effects, in **chapter 4** and **chapter 5**, the endogenous epigenetic environment of the EpCAM and ICAM-1 gene were determined, respectively. Furthermore, the feasibility of re-activating the genes through epigenetic mechanisms is assessed using epigenetic drugs. As such, these studies give information about the potential of using these genes for overwriting repressive

epigenetic marks. In **chapter 4** the molecular epigenetic marks playing a role in regulation of EpCAM expression in ovarian cancer cells are determined. In **chapter 5**, previously suggested epigenetic silencing of ICAM-1 expression in ovarian cancer cells is confirmed for our panel of cell lines. Subsequently, it is investigated in this chapter whether Artificial Transcription Factors, consisting of ICAM-1 specific zinc fingers and the transient activation domain VP64 (four copies of the viral protein VP16), are able to induce gene-expression from the silenced locus. Furthermore, the effect of gene-specific induction of ICAM-1 expression on tumorigenicity is investigated.

In the ovarian cancer cells used in **chapter 4 and 5**, we showed that the EpCAM and ICAM-1 genes are epigenetically silenced. In addition, validated zinc fingers binding these genes are available (59, 61). Therefore, the combination of these genes and cell lines is an interesting model system, uniquely suited to investigate approaches to induce expression of epigenetically silenced genes through Epigenetic Editing. Thus, in **chapter 6**, we attempted to induce expression of the epigenetically silenced EpCAM and ICAM-1 genes in ovarian cancer cells through Epigenetic Editing by targeting putative DNA demethylases or histone modifying enzymes to the target genes.

In **chapter 7**, a general discussion of the results obtained in this thesis follows. In addition, considerations for future research will be presented there.

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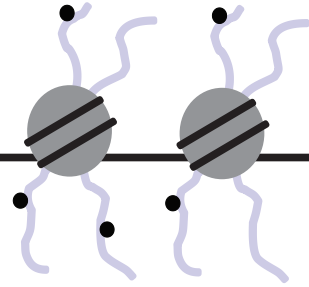
Chapter 2



Epigenetic Editing: Targeted rewriting of epigenetic marks to modulate expression of selected target genes

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ABSTRACT

Despite significant advances made in epigenetic research in recent decades, many questions remain unresolved, especially concerning cause and consequence of epigenetic marks with respect to gene expression modulation. Technologies allowing the targeting of epigenetic enzymes to predetermined DNA sequences are uniquely suited to answer such questions and would provide potent (bio)medical tools. Towards the goal of gene-specific gene expression modulation by overwriting epigenetic marks (*Epigenetic Editing*), instructive epigenetic marks need to be identified and their writers/erasers should then be fused to gene-specific DNA binding domains. The appropriate epigenetic mark(s) to change in order to efficiently modulate gene expression might have to be validated for any given chromatin context and should be (mitotically) stable. Various insights in such issues have been obtained by sequence-specific targeting of epigenetic enzymes, as is presented in this review. Features of such studies provide critical aspects for further improving *Epigenetic Editing*. An example of this is the direct effect of the edited mark versus the indirect effect of recruited secondary proteins by targeting epigenetic enzymes (or their domains). Proof-of-concept of expression modulation of an endogenous target gene is emerging from the few *Epigenetic Editing* studies performed. Apart from its promise in correcting disease associated epi-mutations, *Epigenetic Editing* represents a powerful tool to address fundamental epigenetic questions.

INTRODUCTION

Epigenetics is gaining momentum in nearly all biomedical research fields, and substantial knowledge of the epigenetic marks (e.g. DNA methylation and posttranslational histone modifications) and enzymes involved in reading, writing and erasing of such marks have been obtained (1, 2, 3, 4, 5). The majority of the data reported so far, however, does not provide insights in e.g. the relative importance of the marks with regard to gene expression control, nor in the order of events. In general, the performed studies tend to be descriptive and epigenetic questions related to cause or consequence effects of epigenetic marks with respect to gene expression modulation are under debate (6, 7, 8). In light of the fact that such fundamental questions have only been addressed in a few studies, including (9, 10, 11), targeting epigenetic enzymes to particular chromatin landscapes will provide useful insights. This review sets out to summarize the outcome of targeting approaches with respect to the effect of epigenetic writers and erasers on the chromatin state and/or on gene expression *(Figure 1 and tables)*.

The general principle of DNA-sequence-specific targeting systems is the fusion of (a part of) an epigenetic enzyme to a DNA binding domain (DBD) to enforce the presence of this effector domain on a particular DNA sequence *(Figure 1)*. This target DNA sequence can be located within an oligonucleotide, a plasmid or integrated in a particular chromatin environment in the genome of a cell. In addition to the induced epigenetic changes, the effect of targeting epigenetic enzymes on gene expression can be assessed by measuring gene expression levels of (reporter) genes that lie in close proximity of the DBD recognition site. Most of the reported targeting efforts make use of non-mammalian DBDs and (multiple repeats of) their specific recognition sequences including the yeast Gal4-UAS system (12), the prokaryotic Tet Repressor (TetR)-Tet Operator (TetO) (13), Lac Repressor (LacR)-Lac Operator (LacO) (14, 15) and the LexA repressor-LexA operator system (16). Additionally, some mammalian systems have been exploited to target enzymes to native endogenous chromatin sites, like the Methyl Binding Domain (MBD) of MeCP2 to target enzymes to genomic sites consisting of hypermethylated DNA (17, 18), the DBD of Mixed Lineage Leukemia (MLL) to force epigenetic enzymes to reside on endogenous MLL target genes (19, 20) and the DBD of NFκB to affect NFκB targets (21).

All of the above mentioned systems, however, are somehow limited with regard to addressing biologically relevant questions, because of the need to introduce foreign DBD recognition sites in the host cells, or because they are not specifically targeting one unique site in the genome, but multiple endogenous target sites of the DBD. To specifically bind one genomic address, as explored in Artificial Transcription Factors (ATFs) (22), various classes of DBDs can be engineered, such as designer zinc finger

(ZF) proteins (23), Triplex Forming Oligos (TFOs) (24) and the recently described TALE domains (25). Indeed, with the recent developments in the field of genome editing (where nucleases are fused to sequence-specific ZF proteins to introduce site-specific DNA damage: Methods of the Year 2011 (26)), the targeting of epigenetic editors (writers or erasers) to a specific gene has come within easy reach.

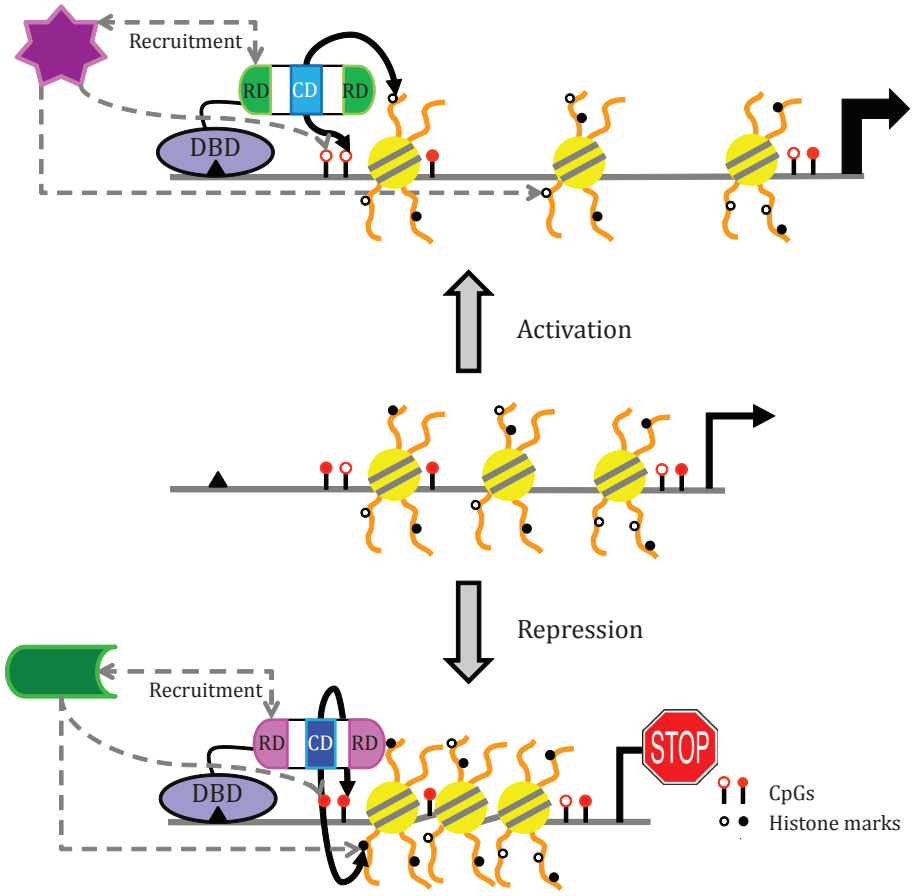

Figure 1. Targeted rewriting of epigenetic marks

Schematic figure, showing the general concept of targeting epigenetic enzymes. In the middle an example of a certain locus harboring a DNA binding domain (DBD) recognition site (black triangle) is shown. Lollypops represent either unmethylated (open) or methylated (filled red) CpGs. Histones (yellow circles) and their tails (orange) are also represented. Histone tails can be posttranslationally modified and as such are associated with a repressed chromatin state (represented by the filled black dots), or with an active chromatin state (represented by open black circles). The upper and lower figure show the induced change in gene expression by targeting a DBD fused to an epigenetic enzyme involved in changing the epigenetic composition (histone modifications or DNA methylation), thereby causing gene activation (top) and repression (bottom). In the epigenetic enzymes, CD=catalytic domains and RD=recruiting domains are indicated. Black arrows show the action of the CD of the epigenetic enzymes, dashed arrows show the possible recruitment of other proteins or capturing by other proteins (Purple star, top; green shape, bottom).

The effective binding of the (gene-specific) DBDs to various euchromatin and heterochromatin targets has been shown by fusing the DBDs to transcription activating or repressive domains (VP64 or SKD, respectively), which recruit other proteins to induce (27) or repress (23) target gene expression. Despite their success (22, 23, 28), ATFs are likely to function only transiently and gene-specific overwriting of epigenetic marks by targeting epigenetic enzymes or domains thereof (*Epigenetic Editing*) might provide advantages with respect to long-term modulation of gene expression. Apart from obtaining sustained gene expression modulation, other advantages of *Epigenetic Editing* include upregulation by allowing natural expression mechanisms to occur, as opposed to mere overexpression as obtained by gene therapy or ATFs. Moreover, the approach of *Epigenetic Editing* is uniquely suited to investigate functions of epigenetic writers and erasers and to elucidate consequences of epigenetic marks at any given chromatin environment, providing insights in gene expression regulation mechanisms. In this review, an overview of studies on sequence-specific and the few gene-specific targeted epigenetic editors will be presented. Also, some points from these studies will be highlighted that will further improve gene-specific *Epigenetic Editing* efforts. Although essential information on chromatin behavior has been obtained by targeting epigenetic readers as well, this review will only focus on direct modulation of epigenetic marks by the implicit targeting of writers and erasers.

GENE EXPRESSION MODULATION WITH TARGETED EPIGENETIC EDITORS

A substantial amount of studies on targeting epigenetic enzymes have provided strong indications that epigenetic editors can be targeted to obtain a change in gene expression. These studies, as discussed in this review, have been using diverse experimental designs ranging from oligonucleotides to endogenous genes in the natural chromatin context. Thus far, the observed effects of targeted epigenetic enzymes (including histone modifying enzymes) have been mainly obtained by co-transfection experiments. Such experiments more closely resemble the endogenous situation than test-tube oligonucleotides experiments, since the fusion proteins (and their target plasmid) encounter endogenous factors that might play a role, or are even required, in changing essential epigenetic marks and/or in obtaining gene expression modulation. However, the use of cotransfections to analyse the effects of targeted histone modifiers is subject of debate. Whereas it has been reported that plasmids are not suitable for establishing the effects of targeting histone modifying enzymes because of the lack of chromatinization (29), others show by ChIP that a molecular effect of targeting histone modifying enzymes can be observed, indicating that histones can get associated with the plasmid (30, 31). These latter studies confirm nuclease-digestion experiments demonstrating the association of nucleosomes on plasmid DNA (32, 33,

34). More informative data, however, can be derived from targeting epigenetic editors to exogenous sites (usually including a reporter gene) integrated in the chromatin context of cells. Although this approach is also artificial and integrated sites are more susceptible to epigenetic silencing, it provides insights in the effect of induced changes on the chromatin context and is suitable to address heritability issues. True *Epigenetic Editing*, where a single endogenous gene is targeted, obviously provides the most relevant information and the few studies published so far will be discussed in more detail.

As described below, although not all of the targeting studies describe effects on both molecular epigenetic level and gene expression modulation, some (including the *Epigenetic Editing* studies) clearly indicate the causal relationship between the rewritten mark and gene expression modulation. Because of differences in experimental design (such as the type of DNA binding domain or design and expression of the construct) it is difficult to compare efficacy of targeted gene expression modulation between studies. It is tempting to speculate that an absence of effect on gene expression upon inducing a change in one epigenetic mark can be explained by the native chromatin context, as exemplified by the protection of DNA for CpG methylation when histone 3 lysine 4 is methylated, which thus might prevent spreading (35, 36, 37). Other marks that remain present in the chromatin context of the targeted gene might recruit enzymes to restore the initial epigenetic profile. In this regard, it is currently unclear if more than one mark needs to be changed to facilitate an effective change in gene expression in the endogenous situation. Furthermore, the change of more than one epigenetic mark could very well be required for heritability of the effect, which would be of importance for therapeutic approaches in particular. Nevertheless, as described in this first part, promising results have been obtained by the active change of just one epigenetic mark and approaches to further improve *Epigenetic Editing* arising from such studies will be discussed in the second part.

Downregulation of gene expression via rewriting of epigenetic marks

In general, targeted epigenetic silencing has advantages over siRNA approaches, which are currently widely exploited for various clinical phenotypes, as reviewed in (38), but which are generally transient and suffer from target-independent effects (39, 40). An alternative (synergistic) approach to downregulate the expression of a gene of interest is by Artificial Transcription Factors, which might proof efficient as only two copies of DNA need to be targeted in every cell, as opposed to numerous continuously produced mRNA molecules. Although significant repression has been achieved for various endogenous genes by fusions of ZFs to the KRAB domain (23, 28), the KRAB domain does not have enzymatic activities by itself and therefore does not directly

interfere with the epigenetic context at the target site. In this respect, direct targeting of epigenetic enzymes to endogenous target sequences (*Epigenetic Editing*) is more relevant, both for biological questions as well as for the potential use of *Epigenetic Editing* as a therapeutic approach in the future. In this section, studies on repressive effector domains targeted to actively interfere with epigenetic marks in order to repress gene expression are discussed.

Targeted DNA methyltransferases

DNA hypermethylation, especially around the transcription start site and exon 1 (41, 42), is strongly associated with inactive genes. Moreover, DNA methylation is in principle faithfully inherited during mitosis, and has been reported to serve as a strong molecular mark for gene silencing memory (43, 44, 45). Therefore, to permanently downregulate the expression of a gene, targeting DNA MTases is an obvious choice. Some alternative gene-specific technologies to induce DNA methylation have been described, including RNA-directed (46) and methylated oligo-induced (47) methylation, but the general applicability of such approaches to silence any gene of interest is unclear. Nowadays, DBDs can be engineered to specifically bind virtually any gene (22, 23) to target transcriptional repressors to these genes, subsequently decreasing gene expression. Indeed, upon fusion of such engineerable DBDs to the KRAB domain, effective reduction of oncogene expression (resulting in reduced tumorigenicity) was shown (28). Thus, fusion of DNA MTases to such domains offers an appealing approach for inducing inheritable gene silencing. In fact, DNA MTases have been extensively studied in fusions to ZFs *(Table 1)*, as also reviewed in (48).

Indeed, upon targeting by fusion to gene-specific (ZFs or TFOs) or sequence-specific (Gal4) DBDs, both the prokaryotic DNA MTases M.*Sss*I, M.*Hha*I and M.*Hpa*II as well as the catalytic domains (CDs) of the mammalian enzymes mDnmt3a and mDnmt3b showed efficient preferential DNA methylation of target sites in oligonucleotides (49, 50, 51, 52, 53) or on reporter plasmids (30, 50, 52, 53, 54) and when assessed, the targeted DNA methylation upon cotransfections was correlated to repression of reporter gene expression *(Table 1)*. The ability of a ZF fused to a prokaryotic DNA MTase to cause preferential DNA methylation at an endogenous mammalian target site was observed for M.*Sss*I in the context of yeast chromatin (55). Noteworthy, the ZF binding site itself was not methylated, indicating protection from direct DNA methylation by the ZF binding. As yeast cells have no endogenous DNA methylation system, targeting specificity can be easily investigated. In this respect, this yeast study –as confirmed in some of the other studies (49, 52, 55)– revealed additional aspecific background DNA methylation, which will be further discussed in '*TOWARDS SPECIFIC GENE EXPRESSION MODULATION - The effector domain'.*

Table 1. Targeted DNA methylation editors

Enzyme	DBD	Target	EGE	GEM	References
M.*Sss*I	ZF	Oligo, endogenous target (yeast)	✓	n.a.	(49, 55)
	TFO	Plasmid DNA (cell free)	✓	n.a.	(50)
M.*Hha*I	ZF	Oligo (cell free)	✓	n.a.	(51, 52, 53)
		Plasmids in bacteria	✓	n.a.	(52, 53)
		Integrated (bacteria)	×	n.a.	(52)
M.*Hpa*II	ZF	Oligo, plasmid DNA (cell free)	✓	n.a.	(51, 52)
		Plasmids/ integrated (bacteria)	✓	n.a.	(52)
		Integrated (mammalian)	✓	↓	(52, 56)
mDnmt3a FL	Gal4	Reporter plasmid (mammalian)	×	↓	(30)
mDnmt3a CD	Gal4	Reporter plasmid (mammalian)	✓	↓	(54)
	ZF	Reporter plasmid (mammalian)	✓	↓	(54)
	ZF	Viral DNA	✓	n.a.	(54)
hDnmt3a CD	ZF	Mitochondrial DNA	✓	n.a.	(59)
		Endogenous target (mammalian)	✓	↓	(60)
mDnmt3b CD	Gal4	Reporter plasmid	✓	↓	(54)
Tet1	Gal4	Integrated (mammalian)	n.a.	↓	(93)
5-MCDG	RXRα-receptor	Integrated (mammalian)	✓	↑	(96)
	LexA	Integrated (mammalian)	n.a.	×	(97)
TDG	NFκB DBD	Endogenous targets (mammalian)	✓	↑	(21)
VP64	ZF	Endogenous target (mammalian)	✓	↑	(98)

×: no effect, n.a.: not assessed, ✓: effect reported, ↓: downregulation, ↑: upregulation

DBD; DNA binding domain, EGE; *Epigenetic Editing*, GEM; Gene expression modulation, ZF; Zinc Finger, TFO; Triple helix Forming Oligo.

To increase specificity of the targeted DNA methylation by prokaryotic enzymes, ZFs were fused to less active mutants of these prokaryotic DNA MTases (M.*Hha*I^{Q237G} and M.*Hpa*II^{F35H}, see also '*TOWARDS SPECIFIC GENE EXPRESSION MODULATION - The effector domain'*) (52). In contrast to ZF-M.*Hha*I^{Q237G}, ZF-M.*Hpa*II^{F35H} was able to induce DNA methylation on its target site, integrated in the bacterial genome. Moreover, when targeting a site integrated in the mammalian genome, ZF-M.*Hpa*II^{F35H} could induce targeted DNA methylation as well as downregulation of the reporter gene (56). Interestingly, the histone modification state of the ZF target site accordingly changed into a repressive state, as an enrichment of H3K9me2 and a reduction of H3K4me3 was observed at the site of the integrated reporter gene where DNA methylation was induced (56). This indicates that active change of one epigenetic mark (in this case DNA methylation) can cause a cascade of changes, which might reinforce the repressive state of the chromatin at the target gene. Such a reinforced repressive chromatin state could explain the observation of remaining DNA methylation at the target site after several cell passages. Moreover, the stable DNA methylation state was associated with stable reporter gene repression at least up till 17 days after the expression of ZF-M.*Hpa*II^{F35H} was no longer detected at both the RNA and protein level (at 6 or 7 days after transfection respectively) (56). This sustained DNA methylation and repression of gene expression is indicative of the DNA methylation induced by ZF-M.*Hpa*II^{F35H} being inherited through cell divisions. This study thus provides proof-of-concept that targeted DNA methylation

can be exploited for sustained gene repression.

Compared with prokaryotic MTases, mammalian DNA MTases, i.e. Dnmt1, 3a and 3b, display several advantages when considered for use in targeted DNA methylation of the mammalian genome: Although, like M.*Sss*I, mammalian DNA MTases can methylate all CpG sequences without any further sequence-restrictions, the low catalytic activity of the mammalian enzymes (57) might better allow restriction of methylation to targeted CpGs by fusion to DBDs. In addition, since being mammalian, the MTase activity is probably not influenced by DNA-histone interactions that theoretically might hamper the prokaryotic DNA MTases, as these originate from histone-less organisms (58). Moreover, mammalian enzymes are more likely to recruit other mammalian proteins important for reinforcement of transcriptional repression. This is nicely exemplified by the Gal4-mDnmt3a full length cotransfection study *(Table 1)*: although no detectable DNA methylation was induced at the target region by this construct, repression of reporter gene expression was observed (30). Recruitment of endogenous co-factors has been observed for this and several other epigenetic effector domains and will be discussed later. Last but not least, the domain will evoke less immunogenicity since it is less foreign to the organism.

The ability to cause preferential DNA methylation at a cellular target site was observed for a ZF fused to hDnmt3a CD, targeting mitochondrial DNA (59). In this study, 23% of the clones analyzed by bisulfite sequencing showed preferential methylation at the cytosine directly adjacent to the ZF binding site (59). Another interesting parameter that can and has been addressed in this study is spreading of the epigenetic mark, which was observed within a region of at least 120 bp surrounding the ZF target site (59). In another study, where DNA methylation was targeted to successfully methylate viral DNA upon cellular infection, spreading up to 380 bp on either site of the DBD recognition site has been observed (54). However, it is not directly clear whether the observed induction of distant DNA methylation in these studies is truly because of spreading or because of the flexibility of the targeting construct or the target DNA.

Only very recently, the first gene-specific ZF-targeted DNA methylation was reported in the nuclear chromatin context for the tumor suppressor gene MASPIN and the oncogene SOX2 (60). Upon this *Epigenetic Editing* approach, via targeting the CD of Dnmt3a to the promoter of the MASPIN gene, the most pronounced targeted DNA methylation (of 50%) occurred for two target CpGs. However, differential positioning of the induced DNA methylation was obtained. The targeted DNA methylation was sufficient to efficiently downregulate MASPIN expression, with up to 90% repression observed in single clones. Efficient repression without dense DNA methylation is in line with other observations, like for p53 where induced DNA methylation of a single specific CpG was shown to severely decrease gene expression (61). Interestingly, the observed

MASPIN downregulation was stably inherited: up to 50 days post-infection, when the expression of the ZF-Dnmt3a CD fusion was barely detectable, gene expression was still repressed. Moreover, treating cells at this time point with the DNA methylation inhibitor 5-azadeoxycytidine released the repression, indicating that the induced DNA methylation is still present. Indeed, methylation patterns remained similar to the patterns observed soon after transductions. Furthermore, knockdown of UHRF1 expression, a protein involved in DNA methylation maintenance, caused significant re-expression of MASPIN. The findings were extended to the oncogene SOX2, which could also be efficiently repressed upon targeting Dnmt3a CD to its promoter by fusion to another ZF. By making use of a doxycycline inducible promoter, expression of the ZF-Dnmt3a CD could be cleared after 48 hrs, allowing the cells to recover from repression. Interestingly, the cells did not recover cell proliferation, again indicating heritability of the repression. Studies like these indicate the benefit of using *Epigenetic Editing* over targeting the KRAB domain.

Targeted repressive histone modifying enzymes

As an alternative (or synergistic) approach to the introduction of DNA methylation at transcription start sites, repression of gene expression can be achieved by targeted modification of histone tail residues. In this respect, chromatin that has a repressive composition is known to be covered by histone H3K9 methylation, exhibiting a chromatin state that is proposed to be able to spread its epigenetic composition for instance via Heterochromatin Protein 1 (HP1)-induced heterochromatinization (62, 63). As such, H3K9 MTases (such as Setdb1, G9a and Suv39H1/SU(VAR)3-9) are of interest to fuse to DBDs for repression of target gene expression. Furthermore, histone H3K27 methylation represents a chromatin state related to Polycomb group protein (PcG) regulated genes that become stably silenced during differentiation and cell fate determination, which makes an H3K27 MTase (like Ezh2 or vSet) another interesting candidate for targeted repression of gene expression. Indeed, H3K9, H3K27 and H3K36 MTases (Set2 and Smyd2) as well as an H3K4 demethylase (LSD1) have been targeted leading to gene repression in all cases where gene expression was assessed *(Table 2a)*. Interestingly, lysine residues like H3K9 and H3K27 can be either acetylated or methylated, and deacetylation of acetylated histone lysine residues is required before the induction of histone methylation on these lysine residues can take place (64). It has long been thought that because acetylation neutralizes the positive charge on the histones, this modification facilitates the open configuration of the chromatin at actively expressed regions in the chromatin (65, 66). Indeed, hypoacetylation is found at promoter sites of genes with low or no expression levels, whereas acetylated histone tails are mainly associated with active genes (64, 67). In addition to this 'charge

hypothesis', acetylation also recruits protein complexes (including chromatin 'readers') by changing the histone modification composition of the chromatin (68, 69). Thus, to silence genes through *Epigenetic Editing*, the HDACs are among the candidate enzymes to be targeted. HDACs of class I (HDAC1, 2 and 3) as well as the sirtuin SirT1 (NAD+ dependent, class III) have been targeted in cotransfection studies using the Gal4 or LexA DBD and indeed reduced gene expression *(Table 2a)*.

Although promising, most studies were not intended to assess the actual induction of the histone mark and no conclusions can be drawn from these studies regarding causal relationships between histone marks and gene expression. In contrast, the anticipated targeted change of histone modifications was assessed and reported for Setdb1 (30) upon cotransfections and for Sirt1 (70), LSD1 (71), G9a (72) and Ezh2 (73, 74) targeted to integrated target sites *(Table 2a)*. Accordingly, these changes in epigenetic marks were associated with repression of gene expression.

Table 2a. Targeted activating histone modifying enzymes

Enzyme	**Aka** (137)	**DBD**	**Target**	**EGE**	**GEM**	**References**
HDAC1		Gal4	Reporter plasmid (in vial)	n.a.	✓	(138)
			Reporter plasmid	n.a.	✓	(139, 140)
HDAC2/		Gal4	Reporter plasmid	n.a.	✓	(140, 141)
RPD3		LexA	Reporter plasmid	n.a.	✓	(112, 142)
HDAC3		Gal4	Reporter plasmid	n.a.	✓	(140)
Sirt1		Gal4	Reporter plasmid	n.a.	✓	(70)
			Integrated (mammalian)	✓	✓	(70)
LSD1		Gal4	Integrated (mammalian)	×	n.a.	(74)
		TetR	Integrated (artificial chromosome)	✓	✓	(71)
Setdb1	KMT1E	Gal4	Reporter plasmid	✓	✓	(30)
G9a	KMT1C	Gal4	Integrated (mammalian)	✓	✓	(72)
		ZF	Endogenous target	✓	✓	(29)
Suv39H1/	KMT1A	Gal4	Reporter plasmid	n.a.	✓	(114, 115)
SU(VAR)3-9		ZF	Endogenous target	✓	✓	(29)
Ezh2	KMT6	Gal4	Integrated (mammalian)	✓	✓	(73)
				✓	n.a.	(74)
vSet		Gal4	Reporter plasmid	n.a.	✓	(109)
Set2	KMT3A	LexA	Reporter plasmid	n.a.	✓	(112)
Smyd2	KMT3C	Gal4	Reporter plasmid	n.a.	✓	(113)

×: no effect, n.a.: not assessed, ✓: effect reported

Aka; also known as, DBD; DNA binding domain, EGE; Epigenetic Editing, GEM; Gene expression modulation, ZF; Zinc Finger.

Interestingly, targeting of Gal4 fused to Sirt1 (70), G9a (72) and Ezh2 (73) to integrated target sites, caused other changes in histone marks in addition to the ones anticipated, as also described for targeted DNA methylation. This again might indicate that the change of one mark can induce a cascade of changes in chromatin modifications which probably reinforces the repressed state and might also add to the persistence of repression. Indeed, mitotically inherited repression of target gene expression was noted at least up to four days after clearance of the tetracycline-inducible expression of

the Gal4-Ezh2 fusion protein in human cells (73), associated with both the anticipated H3K27 methylation and additional H3K4 demethylation. In a murine study, Gal4-Ezh2 did not change other marks than H3K27 methylation (74). In fact, although targeting Ezh2 was shown to recruit Dnmt3a to the integrated target site, no DNA methylation was observed and permissive chromatin marks (H3K4me2, H3Ac) remained present. The absence of DNA methylation, despite the presence of Dnmt3a, might be explained by the presence of H3K4 methylation, as this mark seems to prevent DNA methylation (35, 36, 37) and as such might need to be removed by a specific histone demethylase before repression of gene expression can take place. In this respect, targeting of TetR-LSD1 to an artificial chromosome resulted in demethylation of H3K4Me3 (without affecting H3K9/K27 methylation) and induction of gene expression (71). However, in another study, targeting of Gal4-LSD1 to a target integrated in mammalian cells, with the aim to allow DNA methylation to be induced upon targeting of Ezh2, was not successful (74). It might be that this discrepancy is caused by the chromatin context of the artificial chromosome where LSD1 was targeted to by fusion to TetR, which made it easier to reach the target or to affect the histone modification levels. However, since the experimental designs were so different, it is difficult to compare the two studies.

The crosstalk between histones and DNA methylation (75, 76) has also been described for other histone marks than H3K4 methylation. In this respect, the Histone Methyl Transferase (HMT) G9a, which induces H3K9 methylation, HP1 binding, local heterochromatin formation and gene silencing, can also recruit DNA MTases Dnmt3a and 3b which catalyze *de novo* DNA methylation (77, 78). Similarly, in addition to loss of H3K9 methylation at major centromeric satellites in Suv39h knock-out embryonic stem cells, also a decrease in Dnmt3b dependent CpG methylation has been observed (79). Targeting of such writers might thus result in efficient repression of gene expression. Indeed, gene-specific ZF-targeted histone modifications result in repression of a target gene in the endogenous chromatin context by targeting G9a/Suv39H1 or a histone deimination domain (29, 80). Targeting of G9a or Suv39H1 by fusion to a three-finger ZF designed to bind the gene of interest (VEGF-A), provides the first example of ZF-mediated *Epigenetic Editing* of an endogenous gene (29). This VEGF-A ZF, when fused to a transcriptional activation domain like Viral Protein 16 (VP16) of HSV, caused upregulation of endogenous VEGF-A expression (81) and has been further investigated in phase II clinical trials after fusion to the activator p65. Despite a lack of improved therapeutic effect over placebo treatment (82), these efforts demonstrate the feasibility of targeting genes in a clinical setting by ZFs. Swopping the transcriptional activation domain with the catalytic C-terminal domain of H3K9 MTase G9a (N-terminal 828 amino acids (aa) removed) or with smaller N-terminal deletions (Δ75aa, or Δ148 aa) of Suv39H1, caused induction of at least H3K9me2 as well as repression of the

endogenous target gene (29). The effect of the H3K9 MTases (inducing the anticipated mark and repressing gene expression upon targeting) was validated by us for another endogenous gene by fusing the enzymes to another ZF (83). For VEGF-A, increased levels of H3K9me2 were observed throughout the investigated region, up to 900 bp away from the ZF binding site upon targeting either Suv39H1 (Δ75 aa N-terminal) or the CD of G9a (29). This indicates that the targeting of an H3K9 MTase enables the activation of an endogenous mechanism spreading the H3K9 methylation marks and thereby reinforcing repression. Interestingly, in this study and the one with targeted deimination by Cuthbert et al. (80), the targeting construct was delivered to the cells via transient transfection of an expression plasmid, whereas the only other example of true endogenous *Epigenetic Editing* delivered the construct virally (60). Unfortunately, the observed effects were not followed in time, which would have been interesting because prolonged effects would indicate that the targeted induction of the mark is mitotically inherited.

Induction of gene expression via rewriting of epigenetic marks

Also for induction of gene expression, to reactivate epigenetically silenced genes (for example tumor suppressor genes in cancer), there is a variety of possibilities. The achievements obtained by targeted DNA demethylation, locus-specific addition of acetyl groups to histone tail residues, methylation of H3K4 or H3K79 and demethylation of H3K9 or H3K27 will be discussed in this part (see *Table 1* and *Table 2b*).

Targeted 'DNA demethylases'

To achieve long term re-expression, it seems apparent that the removal of DNA methylation is an important step, at least from around the transcription start site and exon 1 of the target gene (41, 42). However, until quite recently it was not generally accepted that active DNA demethylation occurs in mammals, even though examples had been described of both global and locus-specific active DNA demethylation, as reviewed in (84). In this respect, straightforward mammalian effector domains to obtain targeted DNA demethylation are not available. Now that the concept of active DNA demethylation in mammals is increasingly accepted, efforts to identify mammalian proteins associated with the process of active DNA demethylation resulted in interesting candidates for targeted removal of DNA methylation marks as reviewed in (84, 85). In fact, several mechanisms and proteins were described to be associated with DNA demethylation. In plants, enzymes involved in DNA demethylation are relatively well established. Repressor of silencing 1 (Ros1), Demeter (DME) and Demeter-like proteins (DML2, DML3) are unambiguously associated with active DNA demethylation via base excision repair (BER) in plants (86, 87). Interestingly, although other epigenetic plant enzymes

have been shown to function in a mammalian setting (29), no efforts were reported on expressing or targeting the plant CpG demethylation enzymes in mammalian cells.

Potential mechanisms of active DNA demethylation in mammals, for which some indications have been described, are (1) direct removal of the methyl group, (2) 5meC glycosylation followed by BER (like in plants), (3) deamination followed by mismatch repair and (4) nucleotide excision repair (84, 88). However, most of these mechanisms are still under debate. One mechanism that is now increasingly accepted to play a role in DNA demethylation is oxidation of 5-methylcytosine to 5-hydroxymethylcytosine (5hmC) by the Tet enzymes. The biological role of 5hmC itself is not fully known yet. However, the 5hmC mark has been associated with active genes (89), specifically at the promoter (90) but also in the gene bodies (91, 92). Similarly, ChIP-seq studies demonstrated Tet1 to be present on genes occupied by H3K4me3 (active), H3K27me3 (inactive) or both (bivalent domains) and promoter activity can not be predicted by Tet1 binding (93). For *Epigenetic Editing* purposes, it is noteworthy to mention that 5hmC has been proposed to be an intermediate in the active DNA demethylation pathway (94). Consistent with this, intermediates that might be formed in the process of converting 5hmC to be eventually replaced by an unmodified cytosine were detected recently (95).

Interestingly, upon targeting of Tet1 to five Gal4-binding sites integrated in mammalian cells, repression of the targeted integrated reporter gene was observed (93). Also recruitment of Sin3a, a protein that is part of a transcriptional repression complex could be detected. Unfortunately, this targeting study did not investigate effects on the DNA methylation status of the CpGs of the targeted site. In addition, Tet1 was not targeted to hypermethylated (inactive) genes, so its effects on gene expression in a heterochromatin context are currently largely unknown.

As also suggested in one of the many reviews about DNA demethylation (88), targeting candidate DNA demethylases of the proposed possible pathways to specific genes in different chromatin contexts will provide more insights into the enzyme or enzymes that can truly actively demethylate methylated CpGs. However, only a few studies have employed targeting of potential DNA demethylases so far, of which one not even intentionally: Upon overexpression of 5-MCDG, presently known as Thymine DNA Glycosylase (TDG), the protein associated with the retinoid receptor RXRα (96). A reporter transgene was bound by this receptor and (consequently) DNA demethylation and upregulation of the expression of this gene were observed. Interestingly, TDG was recently fused to the DBD of NFκB, which also led to some DNA demethylation of endogenous NFκB target genes and an increase in target gene transcription (21). Contrastingly, in another study, aiming to prevent silencing, no effect on the expression of an integrated target gene was seen upon stable transfection of LexA-MCDG in

mammalian cells (97).

Alternatively, a ZF targeting study in mammalian cells reported on DNA demethylation induced by targeting a transcriptional activator (VP16-tetramer; VP64) to an endogenous gene (98). Effectively, a gene-specific upregulation of gene expression of 25- to 125-fold on mRNA level was achieved, associated with DNA demethylation of up to 70% of the targeted CpGs. Although it might be suggested that the DNA demethylation is a secondary effect of the VP64-induced transcription, the precise location of demethylation, which is strictly determined by the orientation of the effector domain, would argue against this. Still, it needs to be further investigated whether the DNA demethylation is due to active DNA demethylation by VP64 or its recruited proteins, or whether the effect is merely a consequence of steric hindrance by recruited protein complexes preventing Dnmt1 from copying methylation to the daughter strand upon cell division. In plants, transient transfection of gene-specific ZFs fused to VP64 even resulted in heritable (at least two subsequent generations) activation of gene expression of the targeted gene (99). The sustained effect observed here might also be associated with epigenetic changes like DNA demethylation, although this was not addressed in the specific study.

Targeted activating histone modifying enzymes

Although DNA methylation provides a powerful silencing memory, it is not necessarily a lock for gene expression and many genes have been found to be upregulated despite their DNA hypermethylation status after treatment of cells with HDAC inhibitors as described in (43) and references therein. Thus, instead of (or in addition to) DNA demethylation, gene-specific removal of repressive histone marks and/or induction of activating histone marks might achieve efficient and lasting upregulation of target gene expression. Histone modifying enzymes that are likely to be of interest for obtaining targeted activation of gene expression are histone acetyltransferases (HATs; such as p300, P/CAF, CBP and GCN5) and histone methyltransferases methylating histone tail residues H3K4 or H3K79 (for instance Meisetz and Ash1 or Dot1/Dot1L, respectively). Several 'activating' histone modifying enzymes have been targeted to predetermined target sites (see *Table 2b* for an overview) within reporter plasmids, integrated within host genomes or to endogenous target sites by using the endogenous DBDs MLL or MBD.

Irrespective of the context of the target gene, upregulation of gene expression was seen for most of the activating histone modifying enzymes that were targeted (p300, P/CAF, CBP, GCN5, Meisetz, Ash1 and Dot1). Since most cotransfection studies intended to examine the role of coactivators, molecular chromatin marks were generally not studied (and if so, to a low extent). Importantly, the one cotransfection study that assessed molecular chromatin marks on plasmid level upon targeting of an activating histone

modifying enzyme indeed showed an increase in acetylation by targeted p300 (31).

Interestingly, despite using the same DBD, different genes can be affected when (domains of) other enzymes are fused. Namely, when the HAT domain of CBP in a fusion of CBP to MLL was exchanged for the HAT domain of either P/CAF or GCN5, other genes seem to be upregulated than with the CBP HAT domain, since different (less differentiated) cell surface markers are expressed (20). Thus, this indicates that the various HATs each have their own substrates and/or that depending on the chromatin context different functional effects are induced.

Table 2b. Targeted activating histone modifying enzymes

Enzyme	**Aka** (137)	**DBD**	**Target**	**EGE**	**GEM**	**References**
p300	KAT3B	Gal4	Reporter plasmid	n.a.	✓	(116, 117, 124, 143)
			Reporter plasmid	✓	✓	(31)
		LexA	Integrated (mammalian)	n.a.	✓	(97)
		MBD	Endogenous targets	n.a.	✓	(18)
PCAF	KAT2B	Gal4	Reporter plasmid	n.a.	✓	(124)
		LexA	Integrated (mammalian)	n.a.	✓	(97)
		MLL	Endogenous targets	n.a.	✓	(20)
CBP	KAT3A	Gal4	Reporter plasmid	n.a.	✓	(118, 144, 145, 146, 147)
		MLL	Endogenous targets	n.a.	✓	(20, 148)
GCN5	KAT2A	MLL	Endogenous targets	n.a.	✓	(20)
Meisetz		Gal4	Reporter plasmid	n.a.	✓	(108)
Ash1	KMT2H	Gal4	Integrated (drosophila)	✓	✓	(106)
		LexA	Integrated (mammalian)	n.a.	×	(97)
Dot1/	KMT4	MLL	Endogenous targets	✓	n.a.	(19)
Dot1L		LexA	Integrated (yeast)	n.a.	✓	(110)
JMJD2D	KDM4D	MBD	Endogenous targets	✓	×	(17)
KIAA1718	KDM7A	Gal4	Integrated (mammalian)	✓	✓	(105)

×: no effect, n.a.: not assessed, ✓: effect reported

Aka: also known as, DBD; DNA binding domain, EGE; *Epigenetic Editing*, GEM; Gene expression modulation, ZF; Zinc Finger.

Whereas writing activating marks seems to be effective, removal of repressive marks could also be of interest for activation of genes. In fact, although (tri)methylation marks were long thought to be relatively stable, various enzymes have now been described to actively remove these methylation marks (100, 101). To actively remove the repressive H3K27me3 mark, UTX (102, 103) and JMJD3 (104) could be explored in targeting studies, but to the best of our knowledge no such studies have been reported to investigate the effect of removal of this particular mark. However, other histone demethylases, demethylating H3K9 and/or H3K27me2 (JMJD2D or KIAA1718) have been studied in a targeted fashion and removal of the marks was indeed demonstrated. Targeting of JMJD2D to methylated endogenous genes by fusion to the MBD of MeCP2

only resulted in the intended reduction in H3K9me3 at the analyzed MLH1 gene, no changes in H3K9me2 or DNA methylation levels were observed (17). Upon analyzing the effect on gene expression for this target gene, it appeared that demethylation of H3K9me3 was not enough to induce gene expression (17). Likewise, for another target gene that was assessed (GSTP1), no induction of gene expression could be shown. Molecular marks were not analyzed for this gene. The lack of upregulation despite the change in H3K9 methylation might be explained by the fact that no other (assessed) marks changed. In contrast, targeted KIAA1718 (which did cause upregulation of gene expression) showed an increase of H3 acetylation levels, in addition to the expected decreased level of H3K9me2 (105).

Another example of additional histone modifications changing was observed when targeting drosophila Ash1. Not only H3K4me2 levels decreased, H3K9me2 and H4K20me2 marks were increased at a stably integrated reporter gene in *Drosophila* S2 cells (106). However, the induction of H3K9me2 and H4K20me2 is a known function of dAsh1 in addition to the H3K4 methylation. Interestingly, despite the additional induction of the two marks that are associated with gene inactivity (H3K9/H4K20 methylation), upregulation of the reporter gene expression was still observed. The human homolog of Ash1 was not able to change gene expression upon targeting (97). Nevertheless, this is in line with findings that human Ash1 does not methylate H3K4, but can only mono- and di-methylate H3K36, which might be insufficient for gene expression modulation (107).

As becomes clear from Table 2b, only a few studies addressed the effect of targeting the 'activating' enzymes both on modulation of the histone marks as well as on gene expression, whereas others were not intended to assess both. Studies investigating both of these features can give some insights on whether the cause of activation is the induction of the anticipated mark or merely recruitment of other regulatory proteins (6, 7). From the studies reported so far, indications can be distilled that the anticipated change in histone modification by targeting of p300 (31), Ash1 (106), Dot1L (19), JMJD2D (17) and KIAA1718 (105) indeed led to increase in gene expression of the targeted gene. However, only studies including the targeting of a catalytic inactive counterpart of the domain can firmly indicate a causative relationship between mark and expression regulation and such studies have been performed for DNA methyltransferases (54, 59, 60), Meisetz (108), dAsh1 (106), vSet1 (109), Suv39H1 (29) and G9a (72) as will be described below.

TOWARDS EFFICIENT TARGETED GENE EXPRESSION MODULATION

From the above, one can conclude that targeting epigenetic enzymes is a feasible approach to determine functional domains within epigenetic enzymes and to investigate

the effect of edited epigenetic marks. Various reports also demonstrate that targeting epigenetic writers or erasers indeed affect gene expression levels, and some studies touched upon the chromatin context requirements. So far, three papers on gene-specific *Epigenetic Editing* have been published, describing the targeting of an epigenetic writer to an endogenous locus through fusion to a gene-specific DNA binding domain (29, 60, 80). Indeed, in these studies gene expression was affected, with some indication of spreading of the H3K9me2 mark (29) or mitotic stability of targeted DNA methylation (60). Despite successful attempts on rewriting epigenetic signatures to modulate gene expression, the studies summarized above indicate that many issues remain to be clarified for this approach to become robust. In this respect, questions to be addressed include: a) which epigenetic mark or combination of marks needs to be induced/removed in order to efficiently interfere with gene expression (given a particular chromatin context); b) is the edited mark mitotically stable or will the native epigenetic marks be restored upon removal of the editor; c) what is the influence of the chromatin landscape in determining the outcome. Depending on the envisioned epigenetic change, the most optimal effector domains need to be engineered to selectively, yet efficiently, execute its activity specifically at the targeted site. Some of the studies summarized by us (see Tables) did address such efficiency and specificity issues in more depth and will be discussed below.

Direct gene expression modulation: catalytic activity important

It is subject of a hot debate whether epigenetic marks are the drivers of gene expression regulation or merely associated with expression status (6, 7, 8). Indeed, for some enzymes, targeting studies have shown that introducing mutations in the CD or removal of this domain has little or no effect on the induction of changes in gene expression compared to the effect of their larger or full length counterpart proteins. This indicates that not in all cases catalytic activity of the targeted enzyme is important for an effect on gene expression. Obviously, different studies target the effectors to different chromatin and cellular contexts, and this context will significantly influence the outcome (as described for HATs below). Moreover, differences might also be explained by a variation in expression levels or nuclear entry of the constructs, but this was not addressed in most studies. Despite the fact that it is difficult to distill general rules from the limited amount of studies done so far, some evidence exists that, in particular cases, induction of the mark itself is likely to be sufficient to initiate gene expression differences.

In this respect, targeting of the CDs of Dnmt3a (murine: aa 598-908, human: aa 592-909) and Dnmt3b (murine: aa 557-859) was sufficient to cause targeted DNA methylation and repression of gene expression, whereas targeting of catalytically dead

mutants had no effect on expression levels (54, 59, 60). Also with respect to H3K4me3, replacing a Glycine residue with an Alanine residue at aa position 278 in the catalytic PR/SET domain (aa 246-365) completely abolished the activating potential of Gal4-targeted Meisetz (108). Similarly, mutating the HMTase domain (E1357K or N1458I) of drosophila Ash1 prevented the increase of H3K4 methylation at the target site, and subsequently eradicated the activation of gene expression, as opposed to the wildtype enzyme (106). With respect to the repressive mark H3K27me3, creating a catalytic mutant by changing one aa of the Set domain of the H3K27 MTase vSet (Y105A or Y105F) was sufficient to prevent the decrease in reporter gene expression which was observed upon targeting the wildtype enzyme (109). Also for SUV39H1, the repressive activity of a construct -where the N-terminal 76 aa of the enzyme were deleted- was completely abolished by mutating the catalytic activity (29). In case of Gal4-G9a, a ΔSet construct could not induce H3K9 methylation and subsequently had no effect on gene expression (72). Vice versa, targeting truncated constructs including the HMT activity (aa 210-1202 (72)) of the HMTase G9a and even a domain as small as aa 829-1210 (29) were able to induce H3K9 methylation, and both targeted constructs could efficiently downregulate target gene expression. In general, these studies provide strong indications that epigenetic marks can be instructive in gene expression regulation.

Indirect gene expression modulation: recruitment of secondary enzymes by epigenetic editors

Next to enzymes which seem to rely solely on their catalytic activity for gene expression modulation, other enzymes have been described to possess efficient gene expression modulating activity despite absence of (or mutations in) the CDs. For example for Dot1, the H3K79 MTase from yeast, two mechanisms of derepression can be elucidated, a CD-dependent and a CD-independent mechanism of action. The CD-dependent mechanism was confirmed by targeting of only a small part (aa 172-582: including the HMT domain) of Dot1, which was still able to derepress a target gene integrated in the yeast genome, with similar efficiency as full length Dot1 (110). On the other hand, targeting of only the N-terminal part (1-237 aa, not including the HMT domain) of Dot1 also induced derepression of gene expression, thus via a CD-independent mechanism. Whereas the CD-dependent mechanism works via reducing the binding of Sir proteins (yeast homologs of the mammalian sirtuin HDAC proteins) to the target through methylation of H3K79, the recruitment of the HAT Gcn5 appears to be required for the CD-independent mechanism. In addition, the N-terminal domain seems to function in positioning the gene away from the nuclear envelope (where heterochromatin normally localizes). Similar effects were observed with hDot1L for which several domains were targeted (aa 1-340, 1-430 and 318-430) using the same

model system (110). Although aa 1-430 shows the best effect (better than 1-340), aa 318-430 on its own has no effect. Interestingly, when a truncated part of hDot1L (aa 1-670, including the HMT domain) was investigated in a fusion to MLL (111), less efficient induction of H3K79 methylation was observed when compared with MLL-AF10, which supposedly recruits the full length endogenous Dot1L, confirming that other domains of Dot1L are required for full activity. Therefore, these reports provide an example of an effector domain containing functional domains apart from the CD which also play a role in achieving gene expression modulation.

Similarly, mutating the active site of SirT1 did not abolish all repressive activity. Moreover, deletion of the N-terminal 268 aa (which does not affect the HDAC domain) diminished the repressive effect of SirT1, compared to the full length enzyme (70). This observation might be explained by the fact that the missing part was shown to recruit histone H1b, which has been associated with heterochromatin. Likewise, a fusion of TetR to a catalytically inactive mutant of LSD1 (K661A) was still active, resulting in reduction of H3K4me2 levels at the target site, be it after longer exposure of the target site to the mutant enzyme than for the wildtype (71). The authors suggested that the observed decrease in H3K4me2 levels was a secondary effect due to induced repression of transcription; recruited repressor proteins might in turn recruit the wild type LSD1. Also for Set2, the histone MTase activity can only explain part of the repression, since two mutations (R195G and C201A) in the CD of this H3K36 MTase both did not abolish repression completely (112). It might be that a substantial part of repression by Set2 lacking the active Set-domain is caused by the remaining ability to recruit HDACs, as an association was reported for another H3K36me2 MTase, Smyd2, with HDAC1 and Sin3A (113).

Despite the indications described above that catalytic activity is (at least partially) responsible for the observed effects on gene expression, examples exist of efficient gene expression modulation by targeting enzymes without CDs and/or catalytic activity. Illustrative here is an example of Dnmt3a: although the CD is known to result in DNA methylation and gene repression, the targeting of the full length murine Dnmt3a to a reporter plasmid did not result in DNA methylation (30). Despite this lack of detectable induced DNA methylation, repression of reporter gene expression was observed and, as demonstrated by ChIP, proteins with repressive functions, including Setdb1 and HDAC1, were recruited to the promoter site. Thus, in this case, it seems that silencing of the reporter gene was not caused by DNA methylation through mDnmt3a, but indirectly by recruitment of other repressive proteins. Since the observed repressive complex was also formed on endogenous promoters of hypermethylated genes, as demonstrated by ChIP-reChIP, this study underlines the power of targeting mammalian proteins to

initiate a natural repressive cascade in the mammalian context.

Regardless of the indirect effect on gene expression, the editing of a mark might be an absolute requirement in order for the intervention to be mitotically stable. This is exemplified by a targeting study in which an Ezh2-mutant lacking its catalytic Set domain still efficiently repressed transcription to 10% of the control level (73). As the Gal4-Ezh2 ΔSet fusion protein was not associated with an increase in H3K27me3 levels at the target site, the repression by the ΔSet mutant of Ezh2 might be caused by sterical hindrance through recruitment of binding partners of the Polycomb repressive complex (PRC2) such as EED and Suz12. Confirming this hypothesis, targeting EED to the reporter, using the same system, resulted in gene silencing to 30% of the control level. Moreover, the repressive effect is no longer observed upon clearance of the inducible expression of Gal4-Ezh2 ΔSet. This transient effect is in striking contrast to the effect obtained by targeting wildtype Ezh2 which showed both induction of H3K27 methylation and prolonged repression of reporter gene expression. Most likely, the introduction of H3K27me3 and the subsequent cascade of events, not achieved by the Gal4-Ezh2 ΔSet fusion protein, are essential for obtaining sustained repression.

Theoretically, the presence of certain (recruiting) domains within the effector domain can decrease the efficiency of the targeted enzyme by causing the effector domain to be captured by endogenous proteins before reaching the intended target site. This was suggested to explain the failure to repress endogenous reporter gene expression by targeting full length H3K9 MTase Suv39H1 in fusion to a ZF (29). The intact HP1 interaction domain might be captured by endogenous HP1 proteins in heterochromatin, thereby preventing the fusion protein from reaching its target. Deletion of the N-terminal (HP1 interaction domain containing) 76 aa or 149 aa did result in efficient histone methylation and gene repression of a gene in the chromatin context in a ZF-targeting study (29). Even though the deletion construct could not recruit HP1 directly because it lacked the HP1 interaction domain, H3K9me2 was observed as far as 1 kb upstream of the ZF binding site, which suggests that some spreading did occur. This spreading was mediated by the enzymes recruited by the induced mark itself and not indirectly through recruitment of other proteins by the targeted enzyme, as a catalytic mutant did not demonstrate any enrichment in the methylation marks at this upstream region. Despite the successful repression induced by the truncated Suv39H1, inclusion of parts of the N-terminal domain might help to reinforce the repressive effect as targeting of only this domain (aa 1-195) resulted in equally efficient repression of reporter gene expression compared to targeting of full length Suv39H1 in another study (114). Apparently, in the latter co-transfection study no capturing effect by endogenous HP1 was observed for Gal4-Suv39H1, as the full length could efficiently repress reporter gene expression. Interestingly, deletion of the N-terminal 213 aa of drosophila

SU(VAR)3-9 (of which the first 155 aa are lacking in the human homolog Suv39H1) rendered the Gal4 fusion protein ineffective in a co-transfection study compared to its full length, even though this N-terminal part does not contain the Set domain (115). For the full length enzyme, the strong repressive effect was severely reduced after addition of an HDAC inhibitor, which confirms a role for the interaction of the N-terminal domain with the HDAC RPD3.

The most extensively studied protein with respect to effects of different domains is the HAT p300 (see *Figure 2*), where at least three domains seem to influence gene expression levels. Targeting aa 964-1922 of p300, which includes the HAT domain, by fusion to Gal4 is sufficient to result in activation of reporter gene expression in one study (116), but not in another (117). Also, targeting of a similar but somewhat smaller HAT-containing domain of p300 failed to induce endogenous target gene expression in an MBD targeting study (18). Interestingly, in these latter two studies, p300 fusion constructs without the HAT domain (Δ242-1737 (117) and Δ1472-1522 (18)) could induce activation of gene expression, even to a higher extent than the full length protein. These p300 deletion constructs both contain the N-terminal and the C-terminal activation domains, known to form 'enhanceosomes', which might explain the observed effects. Indeed, targeting of only the N-terminal (aa 1-596, but not aa 1-242) or the C-terminal domain (aa 1737-2414) induced expression, again outperforming the full length fusion construct (117). Differences between effects of similar domains in different studies, however, are observed and might be explained by the location of the targeted sites (as will be discussed in the part '*Towards specific gene expression modulation – The DNA binding domain*'). In this respect, in another study, targeting of only the activating N-terminal 1-596 domain, by fusing it to the MBD of MeCP2, could not induce expression of endogenous methylated genes (18). Of course there are many other factors in addition to the location of the targeted site that are likely to play a role in the observed differences in effects caused by similar domains of the p300 protein. For example, the differences in cell lines and constructs (in particular the DNA binding domains) could determine the controversial outcomes. However, within every study some domains of the p300 protein did show an activating effect, indicating that the experimental set-up was available for effective gene expression modulation to occur by targeted HATs.

Also for CBP, targeting of only the HAT domain and the CBP2 domain (aa 1099-1877) results in gene activation to a higher extent than the full length enzyme (118). Deletion of aa 1458-1475 within the HAT domain eliminated the activating effect of the histone acetyltransferase, even when the CBP2 domain is present. The removal of the CBP2 domain -while leaving the HAT domain intact- resulted in a slightly decreased activating potential. This decrease in activating potential might be explained by the CBP2 domain

containing a number of binding sites for co-activators, although targeting of only the CBP2 domain did not result in an activating effect (118).

In conclusion, for some epigenetic enzymes gene expression modulation is caused through the direct writing or erasing of epigenetic marks, whereas for others recruitment of co-activators or repressors determines functional outcome. However, to achieve efficient, sustained effects (mitotic heritability), the actual editing of epigenetic marks seems warranted.

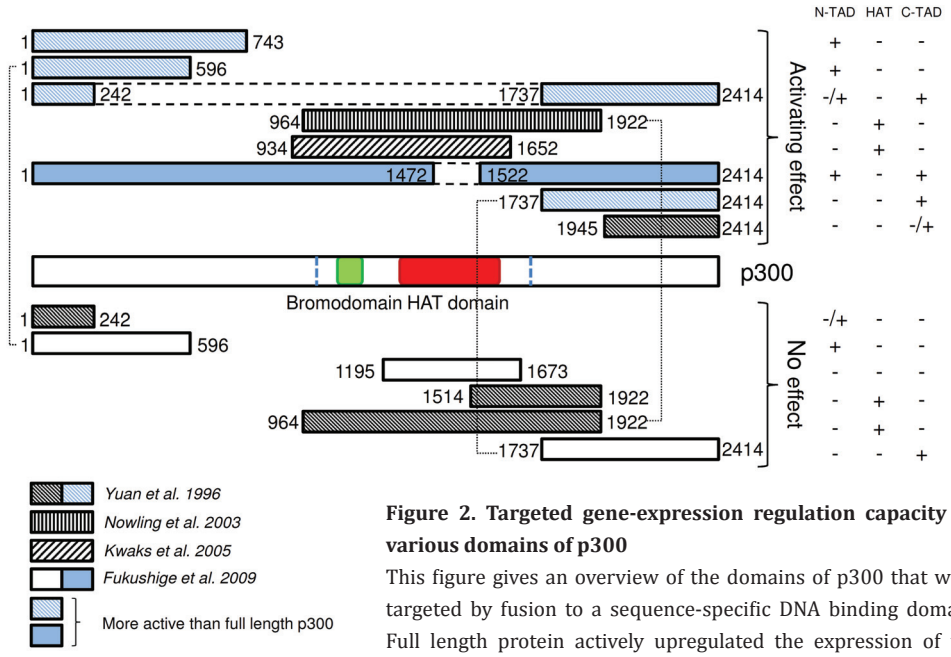

Figure 2. Targeted gene-expression regulation capacity by various domains of p300

This figure gives an overview of the domains of p300 that were targeted by fusion to a sequence-specific DNA binding domain. Full length protein actively upregulated the expression of the target gene in all targeting studies reviewed here. The activating domains are shown above and the non-activating domains below the full length protein. In green and red, respectively, the bromodomain and the HAT domain of p300 are indicated according to a conserved domain search on the NCBI website. Blue dashes in the full length protein show the positions of the commercially available 'HAT domain' of p300 (Millipore). Numbers indicate amino acid positions. Black dashed lines represent a part of the enzyme deleted in the middle of a protein. References are indicated by the pattern of stripes within the domain box as explained in the legend. The table at the right side indicates whether the N-terminal activation domain, HAT domain and/or C-terminal activation domain are present in the related construct. Light blue colou□red domains were more active than the full length enzyme upon comparison within one study. Thin dotted lines connect two equal domains with different outcomes.

TOWARDS SPECIFIC GENE EXPRESSION MODULATION

The effector domain

Thus, targeting epigenetic enzymes or domains thereof, can induce efficient modulation of epigenetic marks resulting in gene expression modulation. Of utmost

importance for general applicability of the approach is the locus specificity of the targeted approach. As exemplified by Gal4-targeted *Drosophila* Ash1, the targeting of domains does not necessarily ensure site specific effects. The natural Ash1 target gene Ubx, which normally is not expressed in the experimental model used, was re-expressed as well in addition to the intended targeted reporter gene (106). The re-expression of Ubx upon expression of Gal4-Ash1 was accompanied by induction of H3K4, H3K9 and H4K20 methylation at this endogenous site as also observed for the intended integrated target site. Apparently, the Gal4 DBD was not strong enough to prevent binding of Ash1 to the Ubx gene. In this line of reasoning, it is also important to realize that certain epigenetic enzymes, such as HATs and HDACs were shown to have an effect on non-histone proteins such as transcription factors (119, 120), which might influence the cell biological outcome.

Similarly, targeting of DNA methyltransferase M.*Hha*I and M.*Hpa*II fused to a four-finger ZF resulted in methylation of their coding plasmids in bacteria although these were devoid of ZF binding sites (52). In addition, background methylation was reported for M.*Sss*I (49, 55). Contrastingly, the enzyme only efficiently functioned on naked DNA when tethered to the DNA; efficient methylation was observed for ZF-M.*Sss*I for oligonucleotides containing the ZF binding site, but not for oligonucleotides without the ZF binding site (49). Although the affinity of M.*Sss*I itself for DNA was decreased upon fusing the enzyme to zinc fingers, it still seems to be too high to allow its site of action to be restricted by DBDs (49). Namely, similar methylation efficiencies were observed for targeted versus untargeted M.*Sss*I in yeast cells, for a non-ZF target locus as well as for the targeted locus (55). Such off-target effects underline the need for MTases strictly functioning at the predetermined target.

A promising way to diminish off-target effects of epigenetic enzymes in targeted fusion proteins is to engineer less active mutants of the enzyme to be fused to the DBD. In this respect, we constructed the M.*Sss*I mutant C141S, with a remaining activity of ~5% of the wildtype activity and conjugated this mutant to a gene-targeting TFO (50). No other CpGs than two targeted CpGs were efficiently methylated in a region of 700 bp of the promoter or in an amplicon of 400 bp investigated within the reporter gene of the plasmid upon co-incubation in a cell-free system, confirming locus-specific DNA methylation. Likewise, diverse mutants of M.*Hha*I and M.*Hpa*II have been constructed (52). For the mutant M.*Hha*I^{Q237G}, which has a remaining methylation activity of less than 5% in *in vitro* enzyme assays, target-specific methylation was confirmed by absence of restriction of the coding plasmid, including the target site, by methylation sensitive restriction enzymes. For M.*Hpa*II, an F35H mutant with reduced activity was created of which the mutated aa normally aids in positioning the adenine ring of *S*-adenosylmethionine (SAM) in the protein binding pocket (52). Also this mutant

shows target-specific methylation in the same system. Evidently, the same approach of creating lower activity/affinity mutants could be used to restrict activity of histone modifying enzymes to the intended loci.

As an alternative to constructing mutant enzymes, the so-called 'split enzyme approach' has been explored to improve the specificity of targeted methylation. Only when the split parts of the protein localize at neighboring sites, the parts of the enzyme can combine and methylate the target sequence. For M.*Hha*I, a plasmid encoding two separate three-finger ZFs fused to complementary halves of the enzyme and also containing the ZF target sites was transformed into *E. coli* (53). Site-specific methylation of the cytosine flanked by the two ZF binding sites was confirmed by bisulfite sequencing. Very recently, another study on targeted split enzymes was reported. Here the M.*Sss*I enzyme was split and recombined to successfully methylate CpGs at the in vivo target site in *E. coli* (121). Although selectivity was not fully supported in another M.*Hha*I split-enzyme study (122), the approach of splitting enzymes (as reviewed in reference (123)) might provide a promising tactic for increasing specificity of *Epigenetic Editing*.

Despite off-target effects, several examples exist where the effect of the targeted enzyme seems to be restricted, since it only takes place when indeed the recognition site of the DBD is present. For example, when targeting human Dnmt3a CD to mitochondrial DNA, no off-target methylation was shown for two distant mitochondrial regions, suggesting usefulness of human Dnmt3a in targeting purposes (59). Likewise, expression of Gal4-CBP had no effect without Gal4 targets being present in the reporter plasmid in a cotransfection study (118).

The DNA binding domain

Off-target effects, on the same chromosome and on other chromosomes, might also be envisioned to occur due to flexible linkers between the DBD and effector domain in combination with dynamic movement of chromatin. Despite the specific binding of the DBD to its unique genomic target site, the effector domain might get in contact with distal sequences due to chromatin folding, but also due to cis and trans interchromosomal interactions. The basic reach-area of epigenetic enzymes has been investigated using oligonucleotides containing DBD recognition sites. In this respect, using oligonucleotides with varying distances (2-32 nucleotides) between the ZF binding site and the target CpG, methylation induced by Zif268-M.*Sss*I was shown to occur preferentially at cytosines 16 or 22 bp upstream of the ZF binding site (49). Although this preference likely reflects the length of the linker between ZF and DNA MTase (19 aa), linker dependence was not further investigated in this study. As for Zif268-M.*Sss*I, the flexibility of the ZF-M.*Hpa*II fusion protein was tested *in vitro* and appeared to be limited, again likely dependent on its linker length (21 aa) (51). A distance of 10 to 40

bps between the ZF target site and the M.*Hpa*II recognition site was most successful for binding and methylation activity of the fusion protein, with optimums at 13 and 34 bps. At 16 and 17 bps distance, a weaker point was detected, which might indicate a position unable to be reached by the effector part of the fusion protein.

Based on the above, the reach area of a ZF-DNA MTase fusion in cell-free systems is limited. Although cellular experiments suggest that recruitment of spreading mechanisms can easily enhance the initial effect, it is of importance to ensure the first hit is efficient. In that respect, it is necessary to know in which direction the effect will have to take place, upstream or downstream of the DBD recognition site, as the site of effect might be determined by the orientation of the effector domain relative to the DBD. For example, for ZF-M.*Sss*I, the direction in which the methylation took place, upstream, was in line with the position of M.*Sss*I in the ZF fusion protein (C-terminal) (49). In another study, for ZF-M.*Hpa*II a preference for methylation of the 3' end of the target site was observed, which is expected because of the orientation of the ZF-M.*Hpa*II fusion protein on the target DNA (52). Also the observed DNA demethylation upon targeting VP64 was in the expected direction (98). Such orientation-dependency, however, might not be observed for all constructs as no clear orientation dependency was observed when targeting a ZF-Dnmt3a CD fusion to an endogenous gene (60).

In addition, most likely it is necessary to modulate more different epigenetic marks to obtain a sustainable effect. As far as known, the effect of targeting two different epigenetic enzymes to the same repeat of DBD recognition sites has not been assessed. However, targeting of vErbA (recruiting the NCoR/SMRT co-repressor complex, executing HDAC activities) and the H3K9 MTase G9a to the same promoter showed an increase of the repressive effect observed compared with targeting either one of the proteins alone (29). Despite that it becomes clear from the above that induction of one epigenetic mark can be sufficient to cause a cascade of events leading to prolonged effects on gene expression, more research into combined targeting of epigenetic writers and/or erasers would be beneficial, especially within the endogenous chromatin context.

Depending on the epigenetic enzyme, it is likely that the genomic site where the effector domain is targeted to (e.g. relative to the transcription start site) is of importance in determining the functional outcome. The target site position at least seems to be playing a role in the case of HATs, as this might explain the contradiction between the two studies targeting p300 aa 964-1922. In one of the studies, the Gal4 binding sites were situated upstream (117) whereas in the other the sites were situated downstream (116) of the transcription start site. Only in the study where the Gal4 binding sites were situated downstream of the transcription start site, an activating effect was seen, as also shown for full length p300 in a different study (124). Fusion of Gal4 to P/CAF again only resulted in upregulation of gene expression after cotransfections when the Gal4

binding sites were situated downstream, not upstream, of the transcription start site of the reporter gene. When the Gal4 binding sites were located upstream, close to the TATA box, an SP1 binding site was needed for reporter gene activation by targeted P/CAF (124).

Evidently, not only the characteristics of the fusion protein, comprising the DBD, the linker and the epigenetic enzyme, influence the specificity and efficacy of the effect. Sensible selection of the target site itself might also be beneficial. In this respect, native chromatin context requirements might be identified in the future which allow efficient *Epigenetic Editing*. From the studies described in this review, it becomes clear that one target site can be sufficient, underlining the feasibility of targeting epigenetic enzymes using a single gene-specific DNA binding domain. Actually, a repeat of target sites is not per se required to improve the effect of histone modifying enzymes, as similar repression has been shown by LexA-RPD3 when the reporter plasmid contained just one LexA binding site when compared to a reporter plasmid with four LexA binding sites (112). For Gal4-Suv39H1, adding an additional Gal4 binding site to the reporter plasmid did not improve the repressive effect either (114).

CONCLUSION

Apart from the ultimate goals of inducing efficient and permanent gene expression modulation, *Epigenetic Editing* is likely to provide valuable insights in cause versus consequence of epigenetic marks with respect to gene expression modulation. It is still a matter of debate whether DNA methylation and posttranslational histone modifications influence the gene expression levels directly or if they are merely byproducts of transcription (6, 7, 125). For example, DNA demethylation was reported to be associated with active histone marks in postmitotic cells, but not with transcriptional activity (126). Targeting minimal CDs of epigenetic writers (and their catalytically dead mutants) to defined chromatin environments allows comparisons determining the effect of the edited mark on higher order chromatin or on gene expression. In this respect, *Epigenetic Editing* might provide a unique tool to eventually settle this cause versus consequence debate.

Before *Epigenetic Editing* can become a straightforward approach, however, the influence of the chromatin context on the dynamics of epigenetics has to be addressed in a systematic manner. It is expected that the positioning of the effector domain as well as the promoter type might affect the ultimate outcome. Similarly, dependent on the cell type, different regulatory protein complexes might be recruited to the same epigenetic mark (127), and different histone (variant) turnover rates and clipping of the histone tails will determine the transient vs. mitotically stable nature of the induced mark (128). In this context, Verschure and collaborators *(manuscript in preparation)* designed a

coarse-grained stochastic model systematically adding epigenetic regulatory levels of increased complexity (i.e. epigenetic enzyme binding, its spreading, subsequent recruitment of regulatory proteins and chromatin folding) allowing to simulate and interpret the mechanistic and dynamic behavior of a nucleosomal stretch to attain a defined epigenetic composition.

Initial publications showed the promise of *Epigenetic Editing* for a handful of genes (MASPIN, Sox2, VEGF-A (29, 60, 80)) and from these studies some indications on heritability (DNA methylation) and spreading (H3K9me) can be distilled. Importantly, these proof-of-concept studies on targeted methylation clearly show the intended repressive effect on gene expression. Although it might be more challenging to effectively compete with spreading mechanisms to overwrite repressive histone marks, accessibility of inactive chromatin presents no limitations as Artificial Transcription Factors have been successful in re-expressing silenced and even imprinted genes (98, 129). With recent developments in the targeted DNA demethylation field (21), combinations of erasers together with certain writers might prove potent in this respect. In fact, *Epigenetic Editing* designed for upregulation of a gene of interest, is advantageous over cDNA approaches as with *Epigenetic Editing* all isoforms can be produced in their natural ratios and expression levels are controlled from the natural promoter and through natural signaling pathways. In addition, silencing via *Epigenetic Editing* through appropriate combinations of marks might prove to be advantageous over approaches like siRNA because the effect would be sustained after clearance of the drug (hit and run approach) (60), without saturating/affecting other cellular (RNAi) processes (39).

With respect to such other gene-specific gene expression modulating approaches (gene therapy or RNA interference), *Epigenetic Editing* promises several advantages, including the broad spectrum of delivery possibilities, ranging from chemical gene-specific epigenetic inhibitors (130) to direct mRNA (28) or protein delivery (27, 131) of the epigenetic editors. Eventually, it will be necessary to achieve efficient, cell- or tissue specific delivery of the *Epigenetic Editing*-device if to be used as therapeutic agent. Although this requires further research, the use of complexes of antibodies recognizing specific cells or tissue and a cationic lipid or liposomes might be powerful approaches to ensure tissue or cell type-specificity (132).

The need for novel epigenetic therapies is exemplified by the numerous ongoing clinical trials to test inhibitors of epigenetic enzymes (133, 134). Although promising, current (FDA approved) epigenetic drugs severely lack specificity not only with respect to the intended target (also unintended non-chromatin proteins are affected), but more importantly with respect to the genome-wide effects (135). The identification of epigenetic marks or combinations of marks which efficiently interfere with gene

expression profiles will open up new avenues in biomedical research. As virtually any (undruggable) gene can be targeted for up- and downregulation (22, 28), *Epigenetic Editing* adds a novel approach to the biomedical arena to investigate gene functions, to validate therapeutic targets and even to be further optimized to become a (synergistic) therapeutic approach (136).

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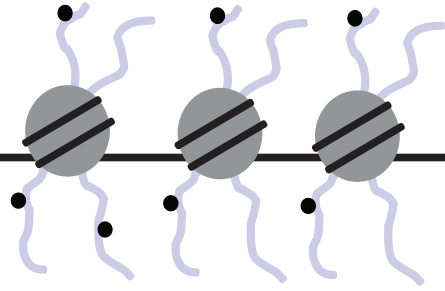
Chapter 3



Screening writers and erasers of epigenetic marks by targeting to predetermined sites to overcome epigenetic silencing

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ABSTRACT

Many diseases are associated with aberrant gene repression which can be caused by epigenetic mutations (including DNA hypermethylation and repressive histone modifications like H3K27me3). As epigenetic marks are potentially reversible, demethylation of DNA or modification of histone tail residues could overcome aberrant repression of gene expression.

In this study, Gadd45α and four other candidate DNA demethylases were investigated for their potency in inducing expression of epigenetically silenced genes. Also the effect of an H3K27 demethylase (UTX) was investigated. The enzymes (or domains thereof) were either overexpressed or targeted to predetermined sites by expressing fusions of the effectors to sequence- or gene-specific DNA binding domains (LacRepressor; LacR or zinc fingers, respectively). Subsequently, gene expression and molecular epigenetic marks were analyzed genome-wide or at the targeted sites. Targeted effects were assessed either on reporter plasmids (LacR and zinc finger) or on large repeats integrated in the genome of cells (LacR).

Out of the five candidate DNA demethylases, Gadd45α was the only one showing slight genome-wide DNA demethylation. Furthermore, significant upregulation of reporter gene expression was observed upon cotransfections of a plasmid encoding untargeted Gadd45α and an *in vitro* methylated luciferase reporter plasmid. Fusion of Gadd45α to a sequence-specific LacR DNA binding domain (targeting to a repeat of LacO sites) or to a gene-specific zinc finger did not result in any significant effect on gene expression or on DNA methylation at reporter plasmids. Nonetheless, an attempt to amplify possible effects by targeting the fusion proteins to a repeat of integrated LacO target sites showed decondensation and an increase of transcription by LacR-Gadd45α when looking at single cell level. Also for and LacR-UTX CD, this effect on transcription was detected.

These results indicate that Gadd45α and UTX likely are able to induce expression of epigenetically silenced genes. Furthermore, indications are obtained that Gadd45α might play a role in DNA demethylation. Epigenetic Editing (endogenous gene-specific rewriting of epigenetic marks) could enlighten the exact role of Gadd45α in gene expression activation. Moreover, the results obtained in this study show that Gadd45α and UTX are interesting candidates to target to endogenous aberrantly epigenetically silenced genes to induce gene expression through Epigenetic Editing.

INTRODUCTION

In many types of diseases, genes have been shown to be aberrantly inactivated not only because of genetic mutations but even more frequently, as becomes more and more clear, because of epigenetic silencing (1, 2). Main features of such epigenetic silencing are DNA methylation (around the transcription start site and exon1 (3, 4)) and the presence of repressive or absence of activating histone modifications (like methylation of histone 3 lysine 27 (H3K27) (5) or hypoacetylated histone tail residues, respectively). Since epigenetic marks are reversible, it is interesting to remove marks associated with repressed genes or add marks associated with active genes to overcome aberrant silencing.

One approach to relieve the repressed state of the chromatin is active DNA demethylation. Although the existence of this phenomenon is accepted by now, the exact mechanism in mammals is still unknown. In plants, the mechanism of active DNA demethylation is well established and described to be performed by Ros1 and DME, bifunctional glycosylase/lyases, which cleave the phosphodiester backbone after they have removed the methylated cytosine, a gap which is subsequently repaired by DNA repair mechanisms (6). In mammals, several potential mechanisms (and proteins involved) have been suggested for active DNA demethylation (7, 8). For example, Gadd45α was implicated to actively demethylate DNA via nucleotide excision repair or base excision repair (9). In addition, Gadd45α was found to associate with deaminases AID and Apobec, as reviewed in (10). Also the DNA methyltransferases 3a and 3b (Dnmt3a, Dnmt3b), known for their capacity to methylate DNA, were described to be able to deaminate methylated cytosines to thymines, in order for the resulting T/G mismatch to be recognized by repair systems, eventually causing DNA demethylation (11, 12).

Another epigenetic mark also often associated with aberrantly repressed genes is H3K27 methylation (13). Therefore, removal of this mark might aid in inducing gene expression. In cells this is known to occur through the activity of histone demethylating enzymes such as UTX (ubiquitously transcribed tetratricopeptide repeat, X chromosome), which specifically recognizes H3K27 methylation (14, 15).

A potent approach to optimally exploit such naturally occurring enzymes is to gene-specifically reverse the repressive epigenetic marks by Epigenetic Editing (16). With Epigenetic Editing, epigenetic enzymes are fused to gene-specific DNA binding domains to execute their effect only at the gene of interest, potentially leading to gene expression modulation. A benefit of this approach, as opposed to (FDA-approved) epigenetic drugs (17), is that it is gene-specific, expected to cause less or no side-effects. Moreover, depending on the epigenetic enzyme fused, both up- and downregulation of genes can be achieved, whereas the epigenetic drugs FDA-approved at present are designed to

only affect epigenetic marks in order to upregulate gene expression. In comparison to other methods developed for activation of gene expression (such as cDNA), one advantage of Epigenetic Editing is that this approach ensures expression of the gene in all isoforms in natural ratios, which was proven to be of importance (18). Moreover, with Epigenetic Editing expression is controlled by the natural promoter. In addition, only two copies of DNA need to be targeted, likely increasing efficiency. For the same reason, also undruggable targets can be easily reached. In addition, epigenetic changes are inheritable (19, 20) and the effect thus can be sustained, whereas the effect of cDNA or siRNA might be cleared upon degradation of the drug.

Towards induction of gene expression by Epigenetic Editing, as aimed for in this study, a number of proteins (supposedly) involved in gene activating epigenetic pathways are screened for both their epigenetic as well as their gene expression modulation effects. In addition to mere overexpression of the proteins to investigate the function of the enzymes, sequence- or gene-specific DBDs are fused to the proteins to tether the enzymes to predetermined DNA target sites in this study. Such targeting not only narrows down the region of investigation, facilitating detectability of the effect, it also enforces the presence of the enzymes at the predetermined target site, whereas overexpressed proteins first need to be recruited to the site of action.

Upon overexpression of the epigenetic enzymes, the effects are assessed at genome-wide level or on reporter plasmids. The DNA binding domains used as targeting tools in this study are the Lac Repressor (LacR) from bacteria, which binds the Lac Operator (LacO) (21) and engineered gene-specific zinc finger proteins (22) (in this case targeting the EpCAM promoter (23)). The fusion proteins comprising these DBDs and the (putative) epigenetic enzymes are cotransfected with *in vitro* methylated reporter plasmids comprising a repeat of LacO sequences or the EpCAM promoter. Moreover, the plasmids encoding LacR fusion proteins are transfected in cells with a large repeat of LacO sequences integrated in their genome (24). It is expected that possible effects of the enzymes are amplified by this system since it enables the targeting of multiple copies of the fusion protein (including the epigenetic enzyme to one target region. Subsequently, molecular epigenetic marks at the target site as well as reporter gene expression levels are analyzed. Furthermore, the effect of the LacR fused (putative) epigenetic enzymes is analyzed in single cells by immunohistochemistry.

MATERIALS AND METHODS

Plasmids

Ros1 catalytic domain (CD; aa 868-1105) and Demeter (DME) CD (aa 1189-1418) were amplified from Arabidopsis cDNA (kindly provided by Dr. B.J. van der Zaal,

University of Leiden, the Netherlands) with forward and reverse primers including BamHI and BglII restriction sites at their 5' end, respectively. pcDNA3.1mycnonHisKozak (pZ; (23)) was restricted with BamHI and the PCR-products were inserted.

UTX CD (aa 401-1401), was amplified from plasmid pGvh0064 (kindly provided by G. van Haaften, NKI, Amsterdam, the Netherlands (25)) with forward and reverse primers containing SalI and XhoI restriction sites at their 5' end respectively. The amplification product was inserted into pZ upon restricting the vector with XhoI.

pcDNA3.1mycHisKozak-EGP2A-Dnmt3a CD (aa 608-908) and pcDNA3.1mycnonHisKozak-EGP2A-Dnmt3b CD (aa 558-859) (kindly provided by Dr. A. Jeltsch) were mutated in their S-Adenosyl Methionine (SAM) binding pocket by site directed mutagenesis to obtain F636A and F587A, respectively. Subsequently, murine Dnmt3a CD F636A and mDnmt3b CD F587A were amplified from these plasmids, UTX and UTX CD from pGvH0064 and Gadd45α from human cDNA, using primers containing AscI restriction sites on the 5' ends. The amplification products were inserted in pU, which was created by insertion of a linker containing AscI restriction sites made by annealing two oligonucleotides: 5'AGCTTCCGCCATGGTTAGATCTCCAAAGAAGAAGAGAAAAGTTACCGGTGGATCCGGCCAGGCCGGCCAGGCGCGCCAGTTAATTAATC 3' and 5'TCGAGATTAATTAACTGGCGCGCCTGGCCGGCCTGGCCGGATCCACCGGTAACTTTTCTCTTCTTCTTTGGAGATCTAACCATGGCGGA 3' into the HindIII and XhoI sites of pZ for untargeted overexpression approaches.

The same amplification products were also inserted into pV, which was (like pV-VP16) created by cutting out LacR from peGFP-LacR (or peGFP-LacR-VP16, respectively (26)) with BamHI and StuI and insertion into pZ which was restricted with XhoI, filled in with Klenow and subsequently restricted with BamHI.

pZ-Up2-Gadd45α was created by restricting Gadd45α from pU-Gadd45α using BglII and XhoI and inserting it into pZ-Up2 (23) which was restricted with BamHI and XhoI.

pCpGL, the luciferase reporter plasmid with a CpG-free backbone was kindly provided by Dr. Michael Rehli (27). To insert the EpCAM promoter, p39E (28) and pCpGL-Basic were restricted with BamHI and the promoter was ligated in the reporter backbone. The pGL3-8.LacO luciferase reporter plasmid (containing 8 Lac Operator) was reported previously (26).

Cell culture

Cos7 cells were maintained in DMEM (BioWhittaker, Walkersville, MD, USA) supplemented with 50 μg/ml gentamicin sulphate, 2 mM L-glutamine and 10% FBS. A2780 cells were maintained in RPMI-1640 medium (BioWhittaker) supplemented with 50 μg/ml gentamicin sulphate, 2mM L-glutamine, 10% FBS, 1mM Na-pyruvaat and 0.05 mM ß-mercapto-ethanol. CHO DG44 AO3_1 cells (24) (kindly provided by Dr. A.S.

Belmont (24)) containing a condensed integration of LacO repeats were maintained in Ham's F-12 medium without thymidine and hypoxanthine, supplemented with triple dialyzed FBS (Perbio) and 0.1 μM MTX, never allowing confluency to reach over 90% or less than 30%. U2os 2-6-3 cells, stably expressing ms2-yfp, were obtained from S.M. Janicki (29) and grown in glutamax DMEM (Gibco, Invitrogen, Life technologies, Bleiswijk, the Netherlands) supplied with 10% tet-approved FCS (Clontech, via Westburg, Leusden, the Netherlands) and 40 μg/ml G418 (Gibco). All cells were cultured at 37°C under 5% CO_2.

As a positive control in DNA demethylation assays, CHO DG44 AO3_1 cells were incubated with different concentrations of zebularine for 48 hrs (as indicated at the figures).

Transfection/profection

Transfection of cells was performed at 60-80% confluency using SAINT-2:DOPE (SD; 0.75mM, Synvolux Therapeutics, Groningen, The Netherlands). 250 ng total plasmid DNA was dissolved in 25 μl HBS and added to 25 μl SD/HBS mixture, consisting of 20 μl HBS and 5 μl SD. This was added to 200 μl serum-free medium and added to one well of a 24 wells plate. 48 hrs after transfection cells were harvested for further analysis. Profection with M.SssI (New England Biolabs, via Bioké, Leiden, the Netherlands) was performed as described before (30). Transfection of the LacR fusion constructs in U2os 2-6-3 cells was performed with Lipofectamin 2000 (Invitrogen), according to the recommendations of the manufacturer.

Genome-wide methylation levels

gDNA was isolated from cells using chloroform/isopropanol precipitation. For methylation sensitive restriction analysis, DNA was either restricted with MspI or with its methylation sensitive isoschizomer, HpaII. Analysis of restriction products was performed on agarose gels.

For LUMA, assays were performed as extensively described in (31, 32). Briefly, gDNA was restricted with EcoRI and HpaII or MspI. Using pyrosequencing, the sticky ends were filled in and quantified using the Pyromark Q24 MD pyrosequencer (Qiagen, Hilden, Germany). Percentage of methylation was calculated by dividing the ratio of filled in cytosines and guanines per adenine upon HpaII digestion by the same ratio upon MspI digestion.

Acid extraction and western blot

Histones were extracted from transfected cells using acid extraction. Cell pellets were dissolved in cold TEB (0.5% Triton-X, 2mM PMSF and 0.2% NaN_3 in PBS) to a density

of 10^7 cells/ml and lysed on ice for 10 min. The lysates were centrifuged for 10 min at 2000 RPM at 4˚C and the pellet was washed with half the volume of cold TEB. After centrifuging again the histones were acid extracted overnight at 4˚C by dissolving the pellets in HCl (0.2 M) at a density of 4.10^7/ml. The next day supernatant (predominantly histones) was collected. To neutralize the samples, 10 µg of histone extract was mixed with 9.5% v/v NaOH (2 M). The samples were separated by SDS-PAGE and blotted on a nitrocellulose membrane. As a loading control, membranes were stained with Ponceau S. Subsequently, membranes were incubated with primary antibody against H3K27me3 (Millipore, Amsterdam, the Netherlands) overnight at 4˚C The next day, the membrane was incubated with goat-anti-rabbit conjugated to alkaline phosphatase (Jackson Immunoresearch, via Sanbio, Uden, the Netherlands) for at least 1.5 hours at RT. To visualize the bands, the membrane was incubated with NBT and BCIP (nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate) substrates.

Western blotting to detect the LacR fusion proteins was performed following the above protocol with regular cell lysates, using a primary antibody (Millipore) against LacR and goat-anti-mouse conjugated to alkaline phosphatase (DAKO, Glostrup, Denmark).

Dual luciferase assay

Dual luciferase assays were performed using the Dual Luciferase assay kit from Promega according to the protocol of the manufacturer. In brief, cells were lysed, and added to LARII reagent. After measurement of Firefly luciferase activity, Stop&Glo reagent was added and Renilla luciferase activity was measured using a Luminoskan Ascent luminometer (Thermo Scientific, Breda, the Netherlands). For calculation of relative luciferase expression, relative light units measured for Firefly luciferase were divided by the relative light units for Renilla luciferase. Subsequently fold induction compared to cotransfections with empty vector were calculated and T-tests were performed to calculate statistical significance.

Plasmid recovery and bisulfite sequencing

For plasmid recovery, cells were first incubated with DNAse (Fermentas, Leon-Rot, Germany) to degrade any plasmid DNA that was not taken up by the cell. Subsequently, plasmid DNA was isolated using the Tip20 kit (Qiagen).

Recovered plasmids were bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo Research via Baseclear, Leiden, the Netherlands) following manufacturers' recommendations. PCR was performed using primers binding the EpCAM promoter, as described before (33). PCR products were extracted from gel using the Qiaquick gel extraction kit (Qiagen) and cloned into the pCR2.1-TOPO vector (Invitrogen). Individual

clones were send for sequencing (Baseclear).

qRT-PCR

Total RNA was isolated using an RNeasy RNA isolation kit (Qiagen). cDNA was synthesized by reverse-transcription using the revertaid cDNA kit (Fermentas). 10 ng of cDNA was used for qRT-PCR on an AB ViiA7 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) using Taqman assays for mGapdH (Mm99999915_g1) and for mDHFR (Mm00515662_m1; both Applied Biosystems). Analysis was performed using the comparative Ct-value method.

Methylated DNA Immuno Precipitation (MeDIP)

gDNA was isolated using chloroform/isopropanol precipitation and subsequently sonicated. The DNA was denatured and dynabeads (Invitrogen) loaded with 5mC antibody (Eurogentec, Seraing, Belgium) or mIgG antibody (Millipore) were added. After overnight incubation, beads were washed and DNA was eluted from the beads. The eluted DNA was purified using a Qiaquick PCR purification kit according to the protocol of the manufacturer. 2 µl of DNA was used in PCR reaction with primers binding to the region right in front of or right behind the extensive LacO repeat.

Chromatin Immuno Precipitation (ChIP)

Transfected cells were fixated using formaldehyde. Subsequently, cells were sonicated to shear the DNA (Bioruptor, Diagenode, Liège, Belgium). Dynabeads loaded with antibodies (mIgG, rIgG, LacR, H3K4me3 and H3K27me3 from Millipore, H3core from Abcam, Cambridge, United Kingdom) were added and incubated overnight. Beads were washed and the DNA/protein complexes were eluted from the beads. Protein and RNA was removed and the DNA was purified and used for PCR like for MeDIP.

Immunohistochemical staining and confocal miscroscopy

Transfected U2os 2-6-3 cells were fixated for 10 min. using 2% formaldehyde. Subsequently the cells were permeabilized by 0.5% Triton-x100, and quenched with 100 mM Glycin. 2.5% BSA was used for blocking and cells were incubated with rabbit and mouse LacR antibodies (obtained from A.S. Belmont) diluted 1:2000 and 1:200, respectively. As secondary antibody, cy3-labeled donkey anti rabbit or mouse (Jackson Immunoresearch) was used, diluted 1:200.

Imaging was performed using a confocal microscope (LSM510, Zeiss, Sliedrecht, the Netherlands). Stacks were taken from 0.2 µm. Optical slices are shown, the bar in the figures represents 5 µm.

RESULTS

Effects of overexpressing untargeted enzymes.

To quantify the genome-wide effect of Gadd45α and mutants of CDs of Dnmt3a and Dnmt3b on methylation levels, LUMA assays were performed upon expression of the constructs in Cos-7 cells (Fig. 1a). The positive control, treatment of the cells with DNA methylation inhibitor zebularine (100 μM), resulted in approximately 5%, but not significant, reduction of genome-wide DNA methylation levels compared to untreated cells. However, a very slight but significant decrease in genome wide methylation levels was observed upon overexpression of Gadd45α (11.6 ± 3.1 %, $p<0.05$) compared to treatment of cells with the transfection agent only. mDnmt3a CD F636A and mDnmt3b CD F587A did not decrease genome wide methylation levels significantly, nor did transfection of the empty vector. Using methylation sensitive restriction analysis, no clear effect of domains of plant DNA demethylases Ros1 and DME was observed either (data not shown).

To investigate the effect of Gadd45α more closely, the plasmid causing overexpression of Gadd45α was cotransfected with a reporter plasmid containing the promoter originating from the EpCAM gene and a CpG-free backbone. The effect of Gadd45α was tested in two conditions; upon cotransfection with the unmethylated reporter plasmid and with the same reporter plasmid which was *in vitro* methylated before transfection. Indeed, reporter expression is decreased to 4 ± 2% upon methylation of the reporter plasmid. Interestingly, overexpression of Gadd45α, compared to transfection with the empty vector, only showed significant upregulation of luciferase expression (4.7 ± 0.9-fold, $p<0.001$) from the methylated reporter, not from the unmethylated one (Fig. 1b). Moreover, after recovery of the reporter plasmid from the cell, bisulfite sequencing showed one out of eleven clones had a stretch of demethylated CpGs in the most upstream region of the two regions investigated. None of the six sequenced clones from the reporter plasmid cotransfected with the empty vector showed significant DNA demethylation (Fig. 1c). The effect was also tested on another *in vitro* methylated reporter plasmid (27 ± 13% luciferase expression compared to the unmethylated reporter plasmid), which does not contain a CpG free backbone, has a different promoter and comprises 8 Lac Operator sequences. Cotransfection of this *in vitro* methylated plasmid with the plasmid encoding Gadd45α also resulted in upregulation of reporter gene expression (1.9 ± 0.4-fold, $p<0.01$) compared to cotransfection with the empty vector (Fig. 1b).

In addition to candidate DNA demethylases, certain histone modifying enzymes might aid in activation of gene expression. To investigate genome-wide histone modification levels upon overexpression of H3K27 demethylase UTX (either full length

or aa 401-1401) in Cos-7 cells, a western blot was performed. Although slight decreases in H3K27me3 seem to be detected upon overexpression of UTX CD, no clear differences in H3K27me3 levels could be detected (Fig. 1d).

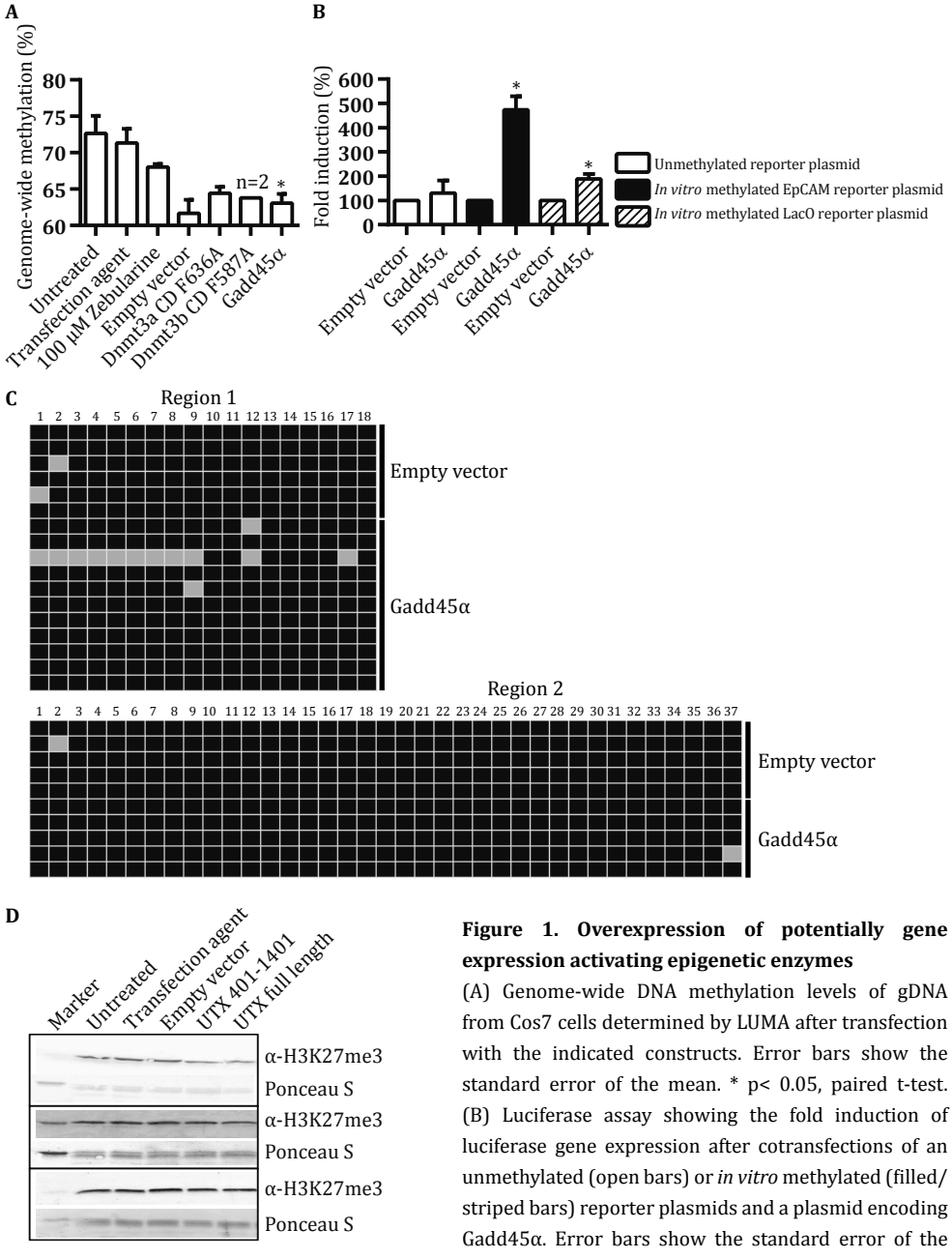

Figure 1. Overexpression of potentially gene expression activating epigenetic enzymes

(A) Genome-wide DNA methylation levels of gDNA from Cos7 cells determined by LUMA after transfection with the indicated constructs. Error bars show the standard error of the mean. * p< 0.05, paired t-test. (B) Luciferase assay showing the fold induction of luciferase gene expression after cotransfections of an unmethylated (open bars) or *in vitro* methylated (filled/striped bars) reporter plasmids and a plasmid encoding Gadd45α. Error bars show the standard error of the mean. * p<0.05, paired t-test. (C) Bisulfite sequencing of recovered reporter plasmids; each box represents a CpG, grey is unmethylated, black is methylated. (D) Western blot to determine genome-wide H3K27me3 levels upon overexpression of UTX or UTX CD.

Specific targeting of candidate DNA demethylases to reporter plasmids

Specific targeting of an enzyme might increase the detectability of the effect. Previously, cotransfections of plasmids encoding Up2-VP64 fusions (targeting the EpCAM promoter) and reporter plasmids containing the EpCAM promoter showed significant upregulation of gene expression (23). Therefore, Gadd45α was fused to Up2 and overexpressed upon cotransfection of its encoding plasmid with the *in vitro* methylated reporter plasmid comprising the EpCAM promoter. Upregulation of reporter gene expression by Up2-VP64 was confirmed for this reporter plasmid with a CpG-free backbone (8.4 ± 0.8-fold, $p<0.05$) compared to cotransfections of the reporter plasmid with the empty vector. Upon *in vitro* methylation of the reporter plasmid before transfection, Up2-VP64 seems to still be able to induce reporter gene expression (5.9 ± 2.1-fold, $p=0.0643$). This indicates that the ZF can bind despite hypermethylation of its recognition site. Data obtained for ZF-targeted Gadd45α, however, are inconsistent (Fig. 2a).

To investigate whether the effect of untargeted Gadd45α (Fig. 1 and Fig. 2a) could be increased by combination with the Dnmt3 mutant enzymes, which are supposed to deaminate methylated cytosine, combined cotransfections were performed. In addition to overexpression of Gadd45α, plasmids encoding fusions of the Dnmt3 mutants to a ZF targeting the EpCAM promotor (EGP2A) were cotransfected with the reporter plasmid containing the EpCAM promoter. No additional effects were observed upon these cotransfections compared to Gadd45α alone (Fig. 2b). Also cotransfections of the reporter plasmid with only the plasmids encoding ZF-fused Dnmt3 mutants did not result in any significant change in gene expression (data not shown).

Targeting of candidate DNA demethylases to a repeat of target sites in a reporter plasmid

To exclude that one binding site for targeting is insufficient, the enzymes were fused to LacR and targeted to a reporter plasmid containing eight LacO sites. First, to be sure that the LacR fusion proteins were indeed expressed, western blots were performed (Fig. 3a). From the western blot of AO3_1 cell lysates it becomes clear that all LacR fusion proteins containing (putative) DNA demethylases are expressed and bands are detected at the expected heights. The LacR-UTX and LacR-UTX CD fusion proteins were not detectable. For LacR-UTX CD a band was detected upon expression of the constructs in Cos7 cells, although the height of the band seems to be higher (~150 kDa) than the predicted band height (74 kDa) (data not shown).

As for the reporter plasmid with the EpCAM promoter, *in vitro* methylation of the reporter plasmid containing eight LacO sites also resulted in efficient repression of gene expression and LacR-VP16, used as a positive control, was able to upregulate luciferase

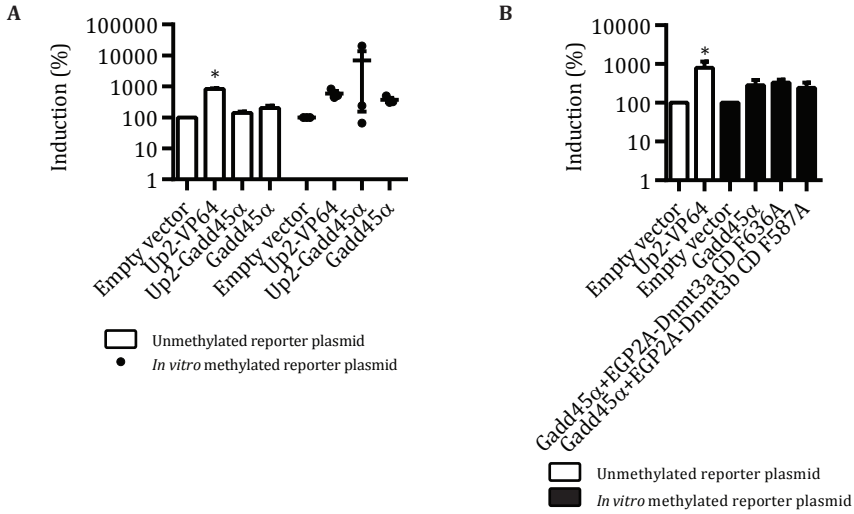

Figure 2. Targeted DNA demethylation to an EpCAM-luciferase reporter plasmid

(A) Luciferase assay upon cotransfections of the indicated constructs with an unmethylated (open bars) or an *in vitro* methylated (black dots) EpCAM-reporter plasmid. For the methylated reporter plasmid, each of the three individual experiments is represented separately with black dots. Error bars represent the standard error of the mean. * $p<0.05$, paired t-test. (B) Luciferase assay upon cotransfections of *in vitro* methylated (filled bars) EpCAM-reporter plasmid with a combination of plasmid encoding untargeted Gadd45α and a plasmid encoding ZF-targeted mDnmt3a/b mutants. Error bars represent the standard error of the mean. * $p<0.05$, paired t-test.

expression (2.3 ± 1.1-fold, $p<0.05$) upon cotransfections with the unmethylated reporter plasmid (Fig. 3b). Moreover, expression of this construct also caused induction of luciferase expression when the reporter plasmid was *in vitro* methylated before cotransfections (2.8 ± 0.9-fold, $p<0.05$), indicating that LacR binding, like ZF binding, is not completely prevented by methylation. However, whereas Up2-Gadd45α gave some indications of being able to induce luciferase expression when targeted to a methylated reporter plasmid, LacR-Gadd45α was not able to do so (Fig. 3b).

Also in this system with more DNA binding domain target sites, targeted (LacR-fused) mDnmt3 mutant enzymes could not further increase luciferase expression compared to untargeted Gadd45α (Fig. 3c). Cotransfection of the reporter plasmid with plasmids encoding the LacR-fused mDnmt3 mutant enzymes alone did not result in a difference in reporter gene expression compared to cotransfection with the empty vector (data not shown).

Targeting enzymes to large endogenous LacO repeats (total cell population analysis)

To investigate effects of the epigenetic enzymes in a situation closer to the natural situation, the enzymes were targeted to an extensive repeat of LacO sequences which

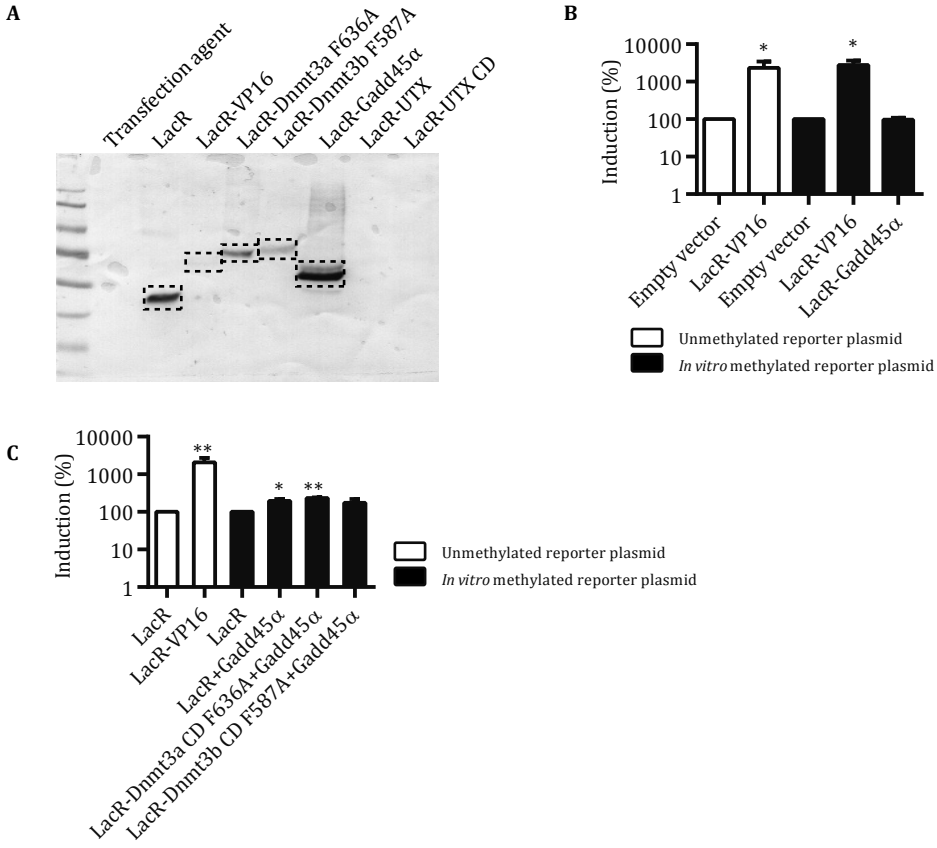

Figure 3. Targeted DNA demethylation at a LacO-luciferase reporter plasmid

(A) Western blot showing expression of LacR fusion proteins, using a LacR antibody. (B) Luciferase assay upon cotransfections of an *in vitro* methylated (filled bars) LacO containing reporter plasmid and a plasmid encoding LacR-fused Gadd45α. Error bars show the standard error of the mean. * $p<0.05$, paired t-test. (C) Luciferase assay upon cotransfections of an *in vitro* methylated (filled bars) LacO-reporter plasmid with a combination of a plasmid encoding untargeted Gadd45 and a plasmid encoding targeted mDnmt3a/b mutants. Error bars show the standard error of the mean. * $p<0.05$, paired t-test.

was integrated in a condensed chromatin region of mammalian AO3_1 cells (24). First, transfectability of AO3_1 cells was established by transfection of a plasmid with the same backbone and promoter (CMV) as the plasmids encoding for the LacR fusion constructs. ~60% of the cells were GFP positive, compared to ~5% for cells transfected with peGFP-LacR-VP16, containing a much weaker promoter (data not shown).

Upon targeting Gadd45α and the mDnmt3 mutants, MeDIP was performed to assess locus-specific DNA methylation levels. Treatment of the cells with zebularine and M.SssI was used as a positive and negative control for DNA demethylation, respectively. It is difficult to perform PCR within the small repeats, as primers will bind in every repeat resulting in multiple products. Therefore, primer sets were designed binding in

close proximity of the start of the LacO repeat or binding just behind the LacO repeat. However, differential DNA methylation was difficult to detect as also with the positive and negative control no visible effect on DNA methylation was obtained (Fig. 4a).

Towards obtaining induction of expression of epigenetically silenced genes, also the histone modifying enzyme UTX CD was targeted to the extensive LacO repeat. The effect of this H3K27 demethylase was analyzed by ChIP, using the same primer sets as for MeDIP (Fig. 4b). However, no clear reproducible effects on histone modification levels could be detected.

To investigate whether targeting of the epigenetic enzymes to the LacO repeat had any effect on expression of the mDHFR reporter gene present in the integrated construct, qRT-PCR was performed (Fig. 4c). 100 μM of DNA methylation inhibitor zebularine was able to induce a slight but significant increase in gene expression (1.4 ± 0.2-fold, $p<0.05$), and 1 mM caused a 1.6 ± 0.3-fold (p 0.0604) increase of mDHFR expression compared to untreated cells. No significant differences were detected upon profection of M.SssI. Also LacR-VP16, without or with eGFP fused to the construct, showed significant increases in expression of mDHFR (2.1 ± 0.3-fold; p 0.0503 and 2.1 ± 0.1-fold; $p<0.05$, respectively). However, in agreement with the assays to determine the change in molecular epigenetic marks, no significant changes in mDHFR mRNA expression were observed upon transfection of the (putative) epigenetic enzymes fused to LacR.

Targeting enzymes to endogenous LacO repeats (single cell analysis)

To by-pass the possibility that low transfection efficiencies cause the lack of effect seen in the AO3_1 cells, we also transfected the LacR fusion constructs in a different model cell line (U2os 2-6-3) where effects can be analyzed at single cell level through confocal imaging (Fig. 5). In this model system, transcripts of a gene in proximity of a repeat of LacO sites are visualized by marking them with a YFP-tag. Furthermore, the LacR fusion proteins are visualized using a secondary antibody with cy3 label which recognizes the LacR antibody. Decondensation of the LacO array as well as an increase in transcription of ms2 is clearly seen for LacR-VP16, as expected. Interestingly, also fusions of LacR to Gadd45α or the catalytic domain of UTX seem to increase expression of ms2 transcripts. The Dnmt3 mutant proteins and full length UTX did not show an increase in ms2 transcripts.

DISCUSSION

In this study, we set out to identify domains which, upon targeting to epigenetically silenced genes, would result in re-expression of that particular gene and methods to identify such domains. We show that out of five different candidate DNA demethylases,

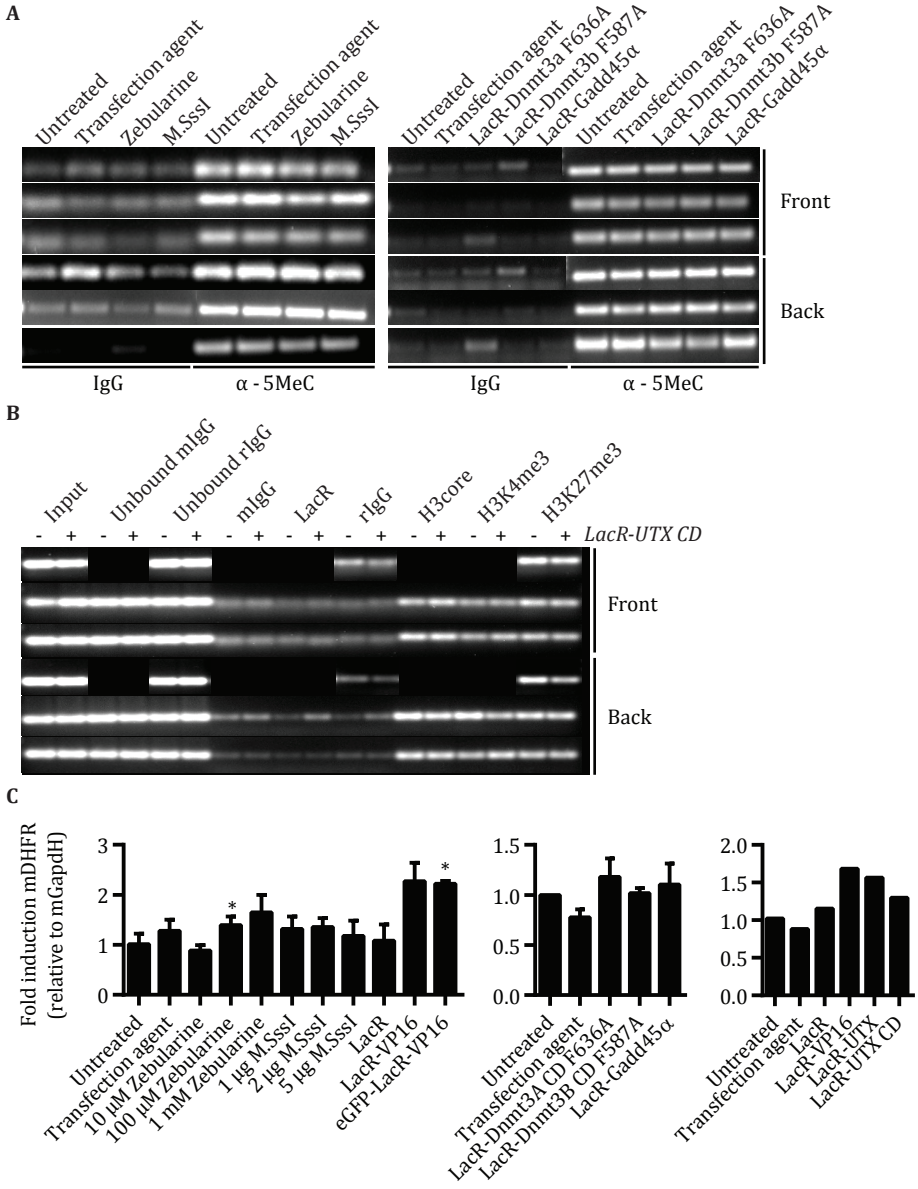

Figure 4. Targeting epigenetic effects to an extensive repeat of LacO sites integrated in a mammalian cell line

(A) MeDIP of two regions in close proximity of the LacO repeat upon treatment with zebularine, profection with M.SssI (left) or transfection with plasmids encoding LacR-fused putative DNA demethylases (right). (B) ChIP of two regions in close proximity of the LacO repeat upon transfection with LacR-UTX CD. (C) qRT-PCR of mDHFR expression upon treatment with zebularine, profection with M.SssI or transfection with LacR-fusion proteins. Experiment with LacR-UTX fusion proteins (figure on the right) is showing the average of two independent experiments. Error bars show the standard error of the mean * $p<0.05$, paired t-test.

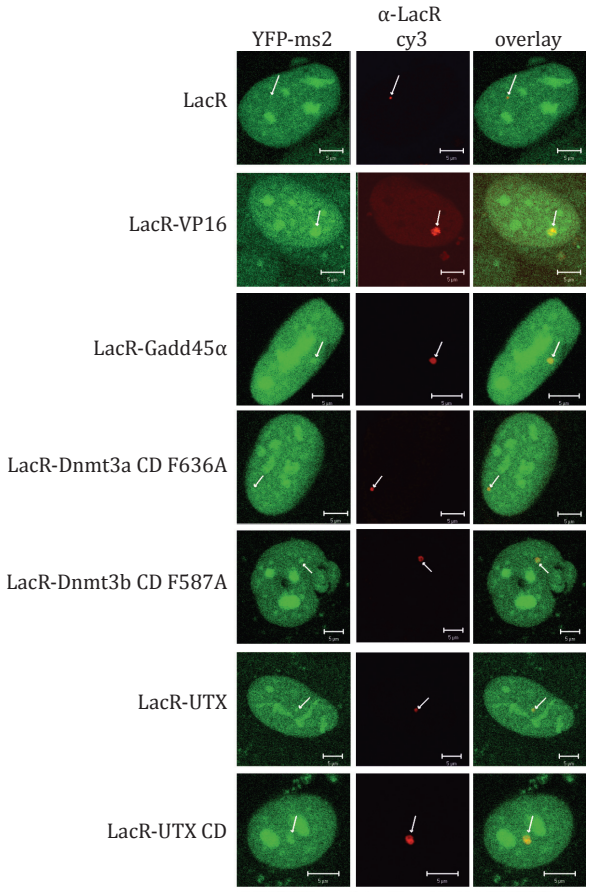

Figure 5. Single cell analysis of the effect of LacR-fused epigenetic enzymes

In this figure, confocal imaging pictures are shown of U2os 2-6-3 cells transfected with the constructs as indicated. The pictures in the left row show yfp-tagged ms2 transcripts. The middle row shows detection of the LacR-fused proteins using a LacR antibody. The row on the right is the overlay of the pictures. Arrows point towards the integrated LacO array. Bars at the bottom of the pictures represent a size of 5 µm.

overexpression of Gadd45α is able to show slight (but significant) genome-wide DNA demethylation. The enzyme is also able to induce gene expression from *in vitro* methylated reporter plasmids upon cotransfections, which is associated with some DNA demethylation. Importantly, when analyzing the effect of LacR fused to Gadd45α at single cell level in cells with an integrated LacO repeat (29), decondensation and an increase in transcription of the targeted reported gene was observed. Also LacR-UTX CD seems to have a positive effect on chromatin decondensation and transcription when analyzed at single cell level.

Gadd45α overexpression caused significant increases in reporter gene expression of 5- or 2-fold, depending on the reporter plasmid used for cotransfections. The highest induction is achieved with a reporter plasmid that does not contain CpGs in its backbone (EpCAM reporter plasmid), whereas the other does (LacO reporter plasmid). It was described before that methylation of CpGs in the backbone and reporter gene of a plasmid can hamper reporter gene expression (27). However, the fold reduction of

luciferase expression upon *in vitro* methylation was less in the LacO reporter plasmid compared to the EpCAM reporter plasmid, which also might (partially) explain the weaker increase in relative expression upon overexpression of Gadd45α. Nonetheless, the extent of induction of reporter gene expression by Gadd45α seems to be in a similar range as described by Barreto et al. where induction was approximately 3-fold (9). Although Barreto et al. could demonstrate that Gadd45α might play a role in active DNA demethylation, the extent of DNA demethylation is difficult to compare with our data since different methods were used. However, in both studies no massive DNA demethylation seems to occur. As bisulfite sequencing only analyzes selected clones, the effect might be underestimated. In this respect, pyrosequencing can be performed to give quantitative results and might be able to detect more significant DNA demethylation.

Intuitively, the effect of an enzyme on a specific locus would be increased upon fusing a locus-specific DNA binding domain to the enzyme. Activation and repression of reporter gene expression by cotransfections was described previously upon fusion of the EpCAM ZFs used in this study to the transcription activator VP64 (four copies of the viral protein VP16) or the repressor KRAB, respectively (23). However, the effect on gene expression upon targeting Gadd45α to the EpCAM promoter of the reporter plasmid by fusing it to the specific ZF was not significant, whereas a significant >4-fold induction was observed upon overexpression of the untargeted enzyme. Interestingly, the effects of untargeted Gadd45α and Up2-VP64 did not reach statistical significance in this experiment (Fig. 2a), in contrast to another experiment (Fig. 1b). So, it might be that, for example, the transfection efficiencies were low in this particular set of experiments.

The lack of improved effects upon targeting Gadd45α specifically to the reporter plasmids could be explained by Gadd45α not being the first step in the DNA demethylation machinery. As such, the effects observed upon untargeted overexpression could be caused by recruitment of Gadd45α by other proteins and/or its recruitment of again other proteins as also indicated in (10). It could be of interest to investigate the other proteins in the complex and it might be beneficial to co-target one of those proteins. As Gadd45α activity was suggested to possibly be associated with deamination of the methylated cytosine (10), we decided to target DNA methyltransferases in combination with the overexpression of Gadd45α. The catalytic domains of the DNA methyltransferases used in this study were previously reported to be active in DNA methylation (34). As the observed role of these DNA methyltransferases in DNA demethylation (11, 12) was suggested to be most effective without the methyl donor (12), we made mutations in the S-Adenosyl Methionine (SAM) binding pocket. The lack of observation of significant genome-wide DNA demethylation effects of the DNA methyltransferases cannot be explained by the fact that in the present study only the catalytic domains of the proteins were used, as these were also used by (12). However, to the best of our knowledge, the

mutant enzyme created in this study was not tested before and might be ineffective in deamination. This could also explain the lack of additional effect upon targeting the protein domains to the reporter plasmids in fusions to ZFs in combination with overexpression of Gadd45α. Further investigation into the deamination mechanism of the DNA methyltransferases might give more indications on creating an effective domain for targeted approaches.

As it might be that the ZF binding site is not located at a position that is interesting for DNA demethylation with regard to reporter gene expression (35) and, moreover, there is only one ZF binding site in the reporter plasmid (good for specificity but perhaps not for efficacy), fusion to LacR and targeting to a repeat of 8 LacO sites in the reporter plasmid was exploited. However, no differences were detected compared to the EpCAM-luciferase plasmid.

An even more extensive repeat of LacO sites has been integrated in mammalian cells (24). Whereas the intention of using the cells with the integrated extensive repeat of LacO sites was to amplify the effect of targeted epigenetic enzymes, some difficulties appeared for detection of the molecular epigenetic effects. Since there is such an amount of repeats of the LacO sites, PCR-based read-outs are difficult because primers will bind in every of the small LacO repeats. This might also explain the lack of detectable effects of LacR-fused UTX CD. A way to circumvent these problems might be through probe-based detection of immunoprecipitated DNA (36).

This LacO/LacR system has proven its utility in visualization of effects on chromatin condensation (24, 26). In this way, fusion of LacR to the activation domain VP16 was shown to lead to decondensation of the chromatin and movement of the chromatin to the interior of the nucleus (37, 38). Expression of the mDHFR gene, which is also present in multiple copies in this system, has never been analyzed before upon targeting a LacR fusion protein to the repeat. Despite the fact that in the AO3_1 cells the repeat is integrated in a condensed chromatin region, DHFR seems to be considerably expressed in the control situation, because of the many copies of the DHFR gene present in the cell line (37). Nonetheless, in this study a slight increase of DHFR expression was still achieved by targeting LacR-VP16 to the repeat of LacO sequences and by treating the cells with zebularine. No difference in DHFR induction seems to occur when using a plasmid with a strong (CMV) promoter and no GFP compared to a plasmid with a weak promoter and encoding GFP-tagged LacR-VP16. Low induction of DHFR expression might be due to transfection efficiency, as is also indicated upon FACS sorting of GFP-positive cells upon expression of the GFP fused LacR-VP16, showing an increased induction of gene expression of about 2.5 times (data not shown).

However, transfectability of AO3_1 cells was established by transfection of a plasmid encoding GFP under the control of the highly active CMV promoter and appeared to

be ~60%, compared to ~5% for peGFP-LacR-VP16. This could also be due to very low levels of expression of GFP in the peGFP-LacR-VP16 construct, which might be below the detection limit. Interestingly, upon visually analyzing the effect of LacR-Gadd45α and LacR-UTX at a single-cell level in U2os 2-6-3 cells, decondensation and an increase of transcription were clearly visible. As these targeted effects could not be detected in total cell populations, this could indicate that transfection efficiency indeed caused an underestimation of the effect in the AO3_1 cells.

In conclusion, we confirm that Gadd45α seems to play a role in gene expression activation, which might be occurring through DNA demethylation. From the results obtained here, however, the other tested enzymes can not be excluded from the list of putative DNA demethylases. In addition, the catalytic domain of UTX is an interesting candidate for re-expressing epigenetically silenced genes. Further research is needed to identify the most potent enzyme or enzymes for activation of epigenetically silenced genes. Epigenetic Editing, the gene-specific rewriting of epigenetic marks at endogenous genes is an interesting and promising tool to study the (sequence of) effects of the enzymes in more detail (16).

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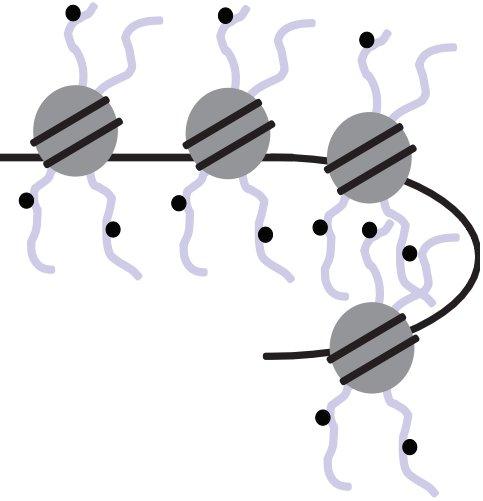
Chapter 4



Transcription factors and molecular epigenetic marks underlying EpCAM overexpression in ovarian cancer

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ABSTRACT

The Epithelial Cell Adhesion Molecule (EpCAM) is overexpressed on carcinomas and its downregulation inhibits the oncogenic potential of multiple tumour types. Here, we investigated underlying mechanisms of EpCAM overexpression in ovarian carcinoma.

EpCAM expression and DNA methylation (bisulphite sequencing) was determined for ovarian cancer cell lines. The association of histone modifications and sixteen transcription factors with the EpCAM promoter was analyzed by chromatin immunoprecipitation. Treatment with 5-Aza-2'-deoxycytidine (5-AZAC) was used to induce EpCAM expression.

EpCAM expression was correlated with DNA methylation and histone modifications. Treatment with 5-AZAC induced EpCAM expression in negative cells. Ten transcription factors were associated with the EpCAM gene in EpCAM expressing cells, but not in EpCAM-negative cells. Methylation of an Sp1 probe inhibited the binding of nuclear extract proteins in electromobility shift assays; such DNA methylation sensitivity was not observed for an NF-κB probe.

This study provides insights in transcriptional regulation of EpCAM in ovarian cancer. Epigenetic parameters associated with EpCAM overexpression are potentially reversible, allowing novel strategies for sustained silencing of EpCAM expression.

INTRODUCTION

The Epithelial Cell Adhesion Molecule (EpCAM; CD326) is a transmembrane glycoprotein, highly overexpressed on most carcinomas. Recently, EpCAM also gained interest as a signal transducer (1) and as a marker of cancer-initiating cells (2). The role of EpCAM in the development of cancer and in tumour progression is dependent on the tumour type as recently reviewed by us (3). For example, in breast cancer high EpCAM expression correlates with poor prognosis (4), and downregulation of EpCAM has been shown to decrease the oncogenic potential (5). In contrast, high EpCAM expression in for example primary renal cell carcinomas is associated with improved patient survival (6, 7). In other types of carcinoma like ovarian cancer, the role of EpCAM is not clear and contradictory results have been reported.

In normal ovary and benign ovarian tumours, EpCAM expression is lower compared to malignant ovarian tumours (8). Numerous studies confirmed the EpCAM overexpression in ovarian carcinomas (9, 10, 11), turning EpCAM into a well established ovarian tumour marker (12). The role of EpCAM in ovarian tumour progression, however, is unclear: one study reported that FIGO stage III/IV showed lower EpCAM expression than stage I (8), while in another study, FIGO stage III/IV showed higher EpCAM expression than stage I/II disease (9). Importantly, metastatic and recurrent tumours were found to express significantly higher levels of EpCAM protein when compared with primary ovarian carcinomas (13). Despite some contradictory results, the observations suggest a promoting rather than a protecting role for EpCAM in ovarian cancer. This promoting role is further confirmed for patients with stage III/IV disease, for whom EpCAM overexpression was shown to correlate significantly with decreased overall survival (11).

Besides its possible prognostic role in ovarian cancer, EpCAM is used as a therapeutic immunotarget for the treatment of malignant ascites. For example, catumaxomab is a trifunctional monoclonal antibody (anti-EpCAM X anti-CD3) approved to treat ovarian cancer patients with malignant ascites (14). Recently, it has been reported that catumaxomab treatment might also have an effect on tumour cells in blood of ovarian cancer patients (15). Similarly, the human monoclonal antibody MT201 could effectively eliminate malignant cells in metastic tumour specimens from patients with ovarian cancer (16).

For various tumour types, EpCAM overexpression has been associated with DNA hypomethylation of the promoter and treatment of EpCAM-negative cells with a DNA methylation inhibitor induced EpCAM expression (17, 18, 19). Alternatively, we also demonstrated that endogenous EpCAM expression can be actively downregulated by nuclear delivery of a DNA methyltransferase (19). Here, we investigate epigenetic mechanisms and transcription factors and underlying the overexpression of EpCAM in

ovarian cancer. Unlike genetic mutations, epigenetic mutations are reversible: a better understanding of the regulation of EpCAM gene expression may thus provide new opportunities for cancer therapy based on reversing epigenetic marks.

MATERIALS AND METHODS

Cell culture and 5-AZAC treatment

Ovarian cancer cell lines (H134S, SKOV3, CaOV3, OVCAR3) were cultured in DMEM (BioWhittaker, Walkersville, MD) and A2780 in RPMI-1640 (BioWhittaker) with 50 µg/ml gentamicin sulphate, 2 mM L-glutamine and 10% FBS. Culture medium of A2780 contained also 1 mM Na-pyruvaat and 0.05 mM β-mercapto-ethanol. For DNA methylation inhibition studies, H134S and A2780 were cultured in their appropriate media with a final concentration of 5 µM 5-Aza-2'-deoxycytidine (5-AZAC; Sigma, St Louis, MO). Every day freshly prepared 5-AZAC was added, and after 3 days cells were harvested for extraction of protein and mRNA.

EpCAM protein expression

EpCAM protein was detected by mouse Mab MOC31 hybridoma supernatant, followed by RαM-F(ab)$_2$-FITC (DAKO, Glostrup, Denmark) or mouse CD326-APC (Biolegend, Uithoorn, Netherlands). The Mean Fluorescence Intensity (MFI) was measured on a Calibur flow cytometer (Beckton Dickenson Biosciences, San Jose, CA).

Quantitative gene expression analysis by Real-Time RT-PCR

RNA was isolated using Rneasy Mini Kit (Qiagen, Venlo, Netherlands), 1 µg was reverse-transcribed (RevertAid cDNA Synthesis Kit, Fermentas, Leon-Rot Germany). Q-PCR was performed (ABIPrism 7900HT, Applied Biosystems, Nieuwekerk, Netherlands) for EpCAM (Hs00158980_m1, Applied Biosystems) and GAPDH (F5'-CCACATCGCTCAGACACCAT-3', R5'-GCGCCCAATACGACCAAAT-3', probe: CGTTGACTCCGACCTTCACCTTCCC (Eurogentec, Maastricht, Netherlands)) in triplicate. Relative gene expression levels were calculated based on the comparative cycle threshold (Ct) method (ΔCt = Ct EpCAM - Ct GAPDH). To compare EpCAM expression of different samples, the differences in ΔCt of individual samples (ΔΔCt) was used (A2780 was set at 1).

DNA methylation analysis

EZ DNA Methylation-Gold Kit (Baseclear Lab Products, Leiden, Netherlands) was used to modify 1 µg of DNA. DNA methylation analysis was performed as described (20). Bisulphite primer sequences for regions A and B are depicted in Fig. 1a. The

correlation between EpCAM expression and DNA methylation was assessed by Spearman correlation test.

Chromatin ImmunoPrecipitation (ChIP)

Histone modifications were determined using antibodies from Upstate Biotechnology (Lake Placid, NY, USA): rabbit IgG, acH4, acH3, H3K4me1, H3K4me3, H3K9me3, H3K27me3, H3K36me2 according to the Upstate protocol with the following modifications. Fixated cells were sonicated using a Bioruptor (High, 15 cycles: 30''on 30''off) (Diagnode, Liège, Belgium). Chromatin fragments were diluted 2.5-fold and precleared for 2h at 4°C. Incubation with antibody was followed by 2h incubation with 60 µl protein A/G-agarose beads. DNA was purified using QiaQuick DNA spin columns (Qiagen, Venlo, Netherlands). To detect association of transcription factors, ChIP was performed as described (21), using the antibodies: mouse IgG, LEF-1(REMB6) TCF (Millipore, Amsterdam, Netherlands), Sp1 (Upstate), STAT3 (Upstate), and from Santa Cruz Biotechnology (Heidelberg, Germany): NF-κBp50(NLS), NF-κBp65(A), ESE-1(H-270), SNAI1(E-130), SLUG(H-140), Ets-1(C-20), Ets-2(C-20), AP2-α(C-18), PEA3(H-120), PDEF(H-250), E2F-2(C-20), E2F-4(C-20) and p53. For the Real Time PCR a freshly made calibration line was included for every primer set used and PCR was finished with a dissociation curve. Conventional PCR was performed according to the protocol of Fermentas (St. Leon-Rot, Germany).

Real Time PCR was performed using Absolute™QPCR SYBRGreenROXMix (Abgene, Surrey, UK), ABI7900HT. The % input was expressed as $AE^{(Ctinput-CtChIP)}$* Fd *100%, where Fd is a dilution compensatory factor and AE represents the primer efficiency. Primers for region A1, B1, B2 and C are depicted in Fig. 1a and underlined in Fig. 1b.

ElectroMobility Shift Assay

OVCAR3 nuclear extract was prepared using an NE-PER kit (Pierce Biotechnology, Etten-Leur, Netherlands). RDY681-labelled probes (Isogen, De Meern, Netherlands) are depicted in Fig. 1b. Probes were incubated with 4 µg nuclear extract in 20 µl binding buffer (Pierce Biotechnology) for 20' at R.T. For competition assays, a 100-fold excess of unlabelled competitor was premixed with RDY681-labelled probe and added to the binding mixture. Probes were *in vitro* methylated by M.SssI (New England Biolabs, Ipswich, MA). Unmethylated probes were treated similarly but in the absence of methyl donor. Nondenaturing 4% polyacrylamide gels were visualised using Odyssey Scanner (Westburg, Leusden, Netherlands).

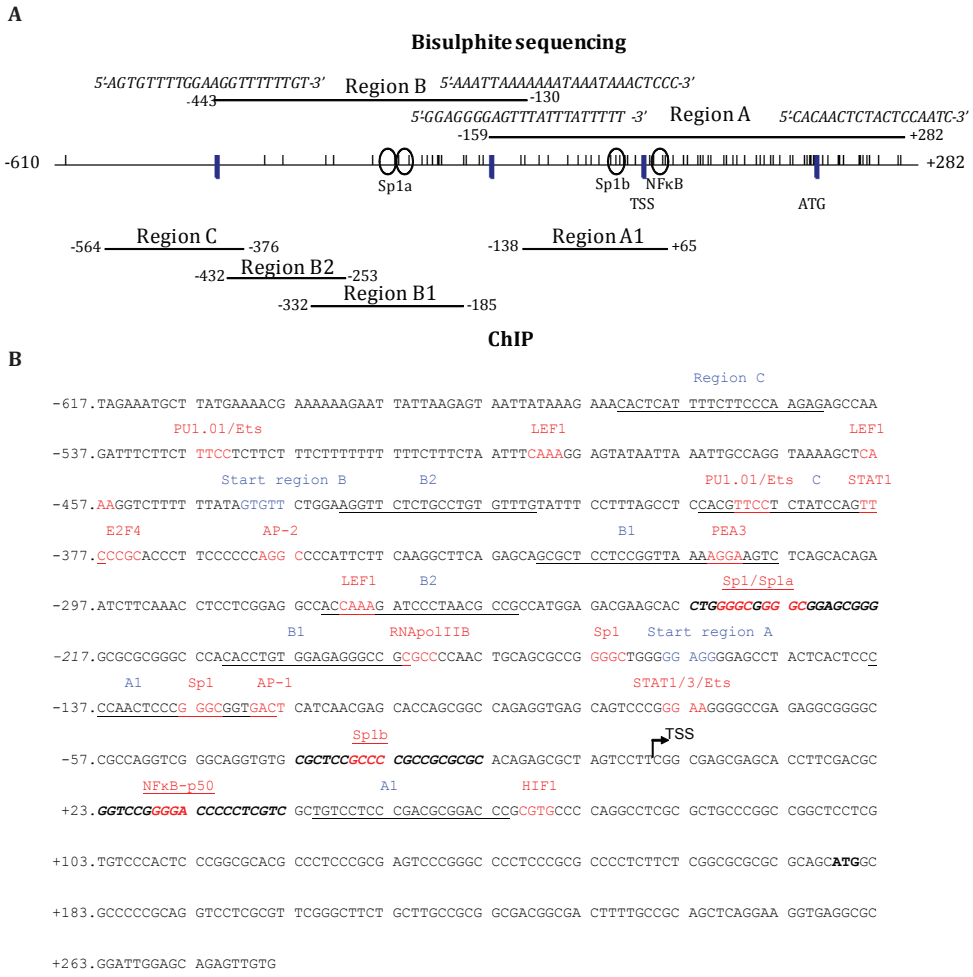


Figure 1. Part of the EpCAM gene under investigation

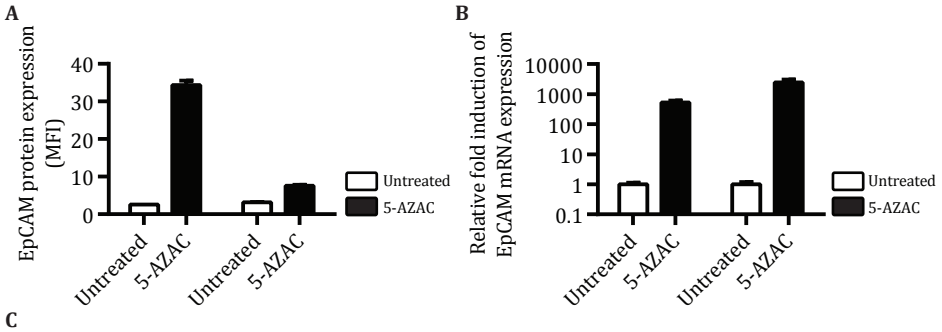
(A) Schematic overview: nucleotide position -610 to +282 relative to the transcription starting site (TSS); the ATG start codon is shown; CpGs are depicted by vertical bars. Region A and B were analyzed for DNA methylation, region C, B2, A1 for histone modifications and region B1 and A1 for transcription factors. Open circles represent putative Sp1 and NF-κB binding sites. (B) Nucleotide positions -617 to +282 relative to the TSS are shown; the ATG start codon is depicted in bold. The start of region A and B are indicated in blue and PCR primers are underlined. Putative transcription factor binding sites analyzed by in silico analysis (Genomatix, MatInspector version 7.7.3.1) are indicated in red. Probes for EMSA to investigate interference of Sp1 and NF-κB binding by DNA methylation are in bold and italic.

RESULTS

EpCAM expression in correlation with DNA methylation in ovarian cancer

Ovarian cancer cell lines were selected based on their EpCAM protein expression

levels: two EpCAM-negative lines (H134S, A2780; MFI: 4.6±0.05, 2.6±0.14 respectively), SKOV3 with an intermediate EpCAM expression level (MFI: 104±3) and two cell lines (CaOV3, OVCAR3) with a high EpCAM expression level (MFI: 461±30; 496±24, respectively) (Fig. 2a). The protein data are in line with the EpCAM mRNA levels (Fig. 2b). To determine the role of DNA methylation in silencing EpCAM expression, the EpCAM negative cell lines were treated with a DNA methylation inhibitor. Indeed, treatment with 5-Aza-2'-deoxycytidine resulted in induction of EpCAM expression in the EpCAM negative cell lines H134S and A2780, both on protein and mRNA level (Fig. 2a and 2b). To further investigate the correlation between EpCAM expression and DNA methylation, the methylation status of the EpCAM promoter and part of exon 1 was analyzed. In the EpCAM-negative cell lines, the 61 CpGs present in region A were hypermethylated (A2780: 100±0%; H134S: 89±23%), whereas region A in EpCAM-positive cell lines was hypomethylated (SKOV3: 1±3%; CaOV3: 0.5±3%; OVCAR3: 0±2%) (Fig. 2c, Table 1). Interestingly, low to undetectable EpCAM expressing normal epithelial ovarian cancer cells (HOSE) (8, 13) displayed a variable DNA methylation level of 15±21% (n = 10 clones). For region B (18 CpGs), the DNA methylation levels were 99±2%, 56±17%, 9±13%, 3±6%, 1±5% for A2780, H134S, SKOV3, CaOV3, OVCAR3, respectively (Fig. 2c, Table I). In the cell lines, an inverse correlation between EpCAM expression and DNA methylation was found (Spearman r = -0.97, p = 0.02, Region A).



C

DNA methylation	A2780	H134S	SKOV3	CaOV3	OVCAR3
Region A	100% (4)	89% (6)	1% (9)	0.5% (6)	0% (6)
Region B	99% (11)	56% (10)	9% (10)	3% (10)	1% (10)

Figure 2. EpCAM expression in correlation with DNA methylation in ovarian cancer

To compare EpCAM expression between the different cell lines, untreated A2780 was set at 1. A) The average (±SD) of the relative Mean Fluorescence Intensity of one representative staining performed in triplicate is shown. B) Quantitative RT-PCR analysis showing relative EpCAM mRNA levels C) The % of DNA methylation represents the number of methylated CpGs divided by the total number of CpGs present in the region. For each cell line the number of clones analyzed is indicated between brackets.

Table 1. Epigenetic marks associated with EpCAM expression

	A2780	H134S	SKOV3	CaOV3	OVCAR3
EpCAM expression	-	-	+	++	++
active histone marks Region C/B2					
acH4	-	-	+	+	+
acH3	-	-	+	+	+
H3K4me3	-	-	+	+	+
active histone marks Region A1					
acH4	-	-	++	++	++
acH3	-	-	++	++	++
H3K4me3	-	-	++	++	++
repressive histone marks Region C/B2					
H3K9me3	-	-	-	-	-
H3K27me3	-	-	-	-	-
repressive histone marks Region A1					-
H3K9me3	+	-	-	-	-
H3K27me3	-	+	-	-	-
DNA methylation Region B/A	+++	++	+/-	-	-

Histone modifications associated with EpCAM expression

In EpCAM-positive cells, region C and B2 were associated with acetylated histone 4 (acH4), acetylated histone 3 (acH3) and with trimethylation of lysine 4 of histone 3 (H3K4me3) (Fig. 3a and 3b, Table 1). For region A1 covering the TSS, the presence of these active marks was even more pronounced (Fig. 3c). In EpCAM-negative cells, association of these histone modifications was not detected, except for low levels of acH3 up to 1% of input DNA at region A1 (Fig. 3d).

The repressive histone modifications H3K9me3 as well as H3K27me3 were not detected in EpCAM-positive cells. Interestingly, in the EpCAM-negative cells region A1 was associated with repressive marks: in A2780, region A1 was associated with H3K9me3; whereas in H134S the promoter was associated with H3K27me3 (Fig. 3d, Table 1).

In vivo EpCAM gene occupancy by transcription factors

Locations of transcription factor binding sites in the EpCAM promoter as described in literature (22, 23, 24, 25), as well as additional putative sites obtained by Genomatix MatInspector are shown in Figure 1b. The transcription factors screened for *in vivo* association with the EpCAM promoter were selected based on evidence for a biological role in EpCAM regulation (18, 24, 25, 26) and their potential role in ovarian cancer (27, 28, 29, 30).

In the EpCAM-positive OVCAR3 cells, the promoter was associated with Sp1, NF-κB,

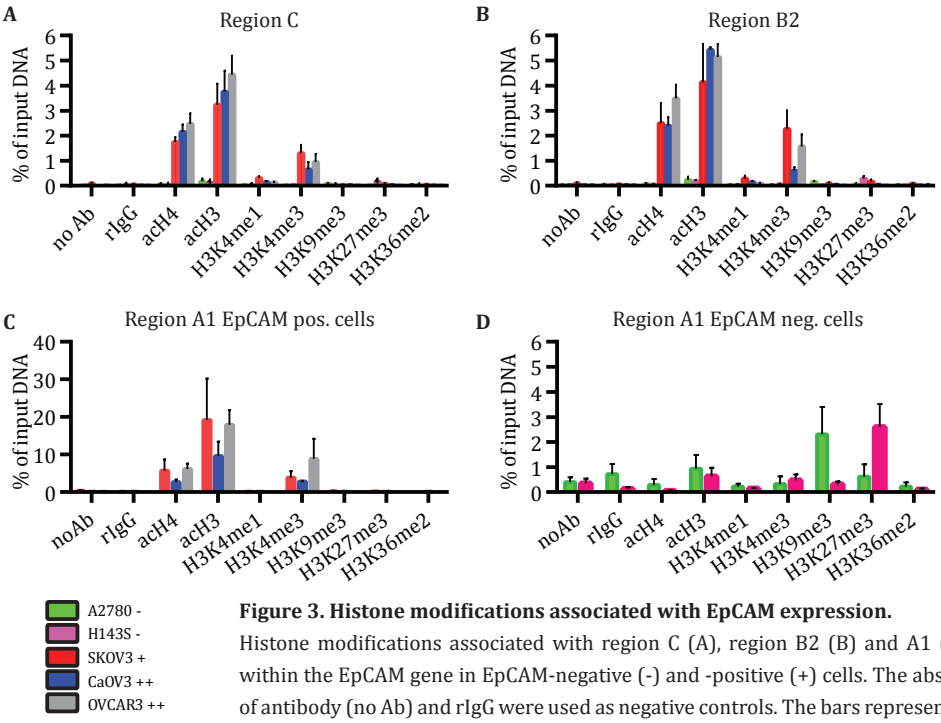


Figure 3. Histone modifications associated with EpCAM expression.
Histone modifications associated with region C (A), region B2 (B) and A1 (C,D) within the EpCAM gene in EpCAM-negative (-) and -positive (+) cells. The absence of antibody (no Ab) and rIgG were used as negative controls. The bars represent the mean of 3 or more independent ChIP experiments ± the SEM.

LEF-1, E2F2, Ets-1 and Ets2 for both regions tested (Table 2 and Fig. 4), whereas E2F4, p53, AP-2α and STAT3 were only associated with region B1. In the EpCAM-positive CaOV3 cells, the promoter was associated with the same transcription factors as for OVCAR3, except that for p53 and STAT3 no association was detected. The transcription factors LEF-1 and Ets1 were associated with region A1, whereas association of Sp1, E2F2, Ets2 and again AP-2α were only found in region B1. In the EpCAM-negative cells A2780 and H134S, no association of any of the transcription factors with region A1 nor with region B1 was detected (Table 2). In addition, no association of ESE-1, SNAI1, SLUG, PEA3, and PDEF was detected in EpCAM-positive nor in EpCAM-negative cells (data not shown).

Interference on binding of transcription factors by DNA methylation

The ChIP data suggest a role for NF-κB and Sp1 in regulating EpCAM gene expression. Our bisulphite sequencing revealed that the CpG next to the putative binding site of NF-κB (located at +27, NF-κB in Fig. 1b) was methylated in all clones of the EpCAM-negative cells and not methylated in the EpCAM-positive cells. Similarly, for the CpG present in a putative binding site for Sp1 (located at -32 , Sp1b in Fig. 1b), complete methylation in all clones was observed in the EpCAM-negative cells, whereas in the EpCAM-positive cells this particular CpG was not once methylated. Also the 2 CpGs present in two

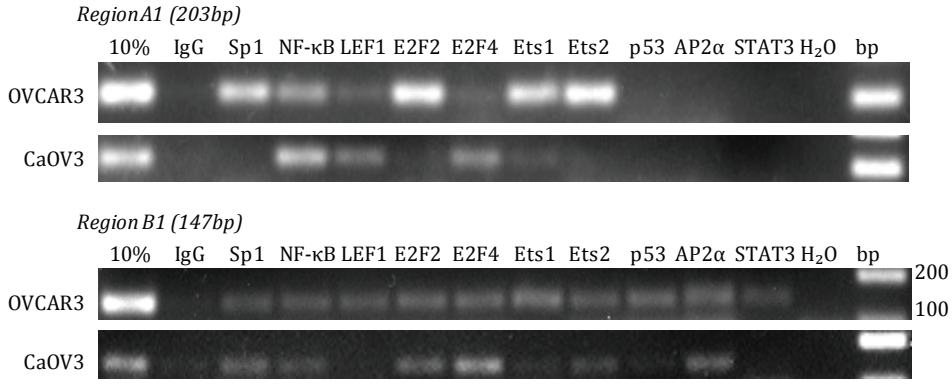


Figure 4. Transcription factors associated with the EpCAM gene.
ChIP analysis on EpCAM-positive (OVCAR3, CaOV3) cells performed with the indicated antibodies, IgG was used as a negative control, PCR was performed with primers for region A1 and B1.

putative binding sites for Sp1 (located at -231 and -226, Sp1a in Fig. 1b), were both methylated in the EpCAM-negative cells (A2780: 22/22 clones, H134S: 14/20 clones), whereas in the EpCAM-positive cells, these two CpGs were not methylated (SKOV3 and CaOV3: 1/20 clones, OVCAR3: 0/20 clones).

To investigate whether the observed DNA methylation actually interferes with binding of the transcription factors to the EpCAM promoter, EMSA competition studies were performed. Shift assay with unmethylated probe Sp1a (Fig. 1b) and nuclear protein extract of OVCAR3 cells revealed two bands (a+b) (Fig. 5a). Both bands were also observed for the methylated Sp1a probe, but the binding of nuclear protein to the methylated probe was less efficient than to the unmethylated probe (lane 2 compared with 6). Also competition with an excess of cold unmethylated Sp1a probe was more efficient than with a methylated probe, indicating that Sp1 binds preferentially to the unmethylated Sp1a binding site within the EpCAM promoter. Shift assay with the Sp1b probe and nuclear extract of OVCAR3 cells revealed two bands with the unmethylated

Table 2. Trancription factors associated with the EpCAM gene (+ = association, - = no association)

		Sp1	NFκB	LEF-1	E2F2	E2F4	Ets1	Ets2	p53	AP2α	STAT3
OVCAR3	A1	+	+	+	+	-	+	+	-	-	-
	B1	+	+	+	+	+	+	+	+	+	+
CaOV3	A1	-	+	+	-	+	+	-	-	-	-
	B1	+	+	-	+	+	-	+	-	+	-
A2780	A1	-	-	-	-	-	-	-	-	-	-
	B1	-	-	-	-	-	-	-	-	-	-
H143S	A1	-	-	-	-	-	-	-	-	-	-
	B1	-	-	-	-	-	-	-	-	-	-

probe as well as with the methylated probe (Fig. 5b). One of the bands is not specific (N.S.) since the band intensity was not reduced with an excess of competitor. The other band indicated with an S, showed competition with both an excess of unmethylated as well as an excess of methylated probe, indicating that for this particular sequence the transcription factor binds to the Sp1b probe regardless of DNA methylation status of the CpGs within this probe. Also for the NF-κB probe, no difference in binding patterns to the methylated and unmethylated NF-κB probes was observed (data not shown).

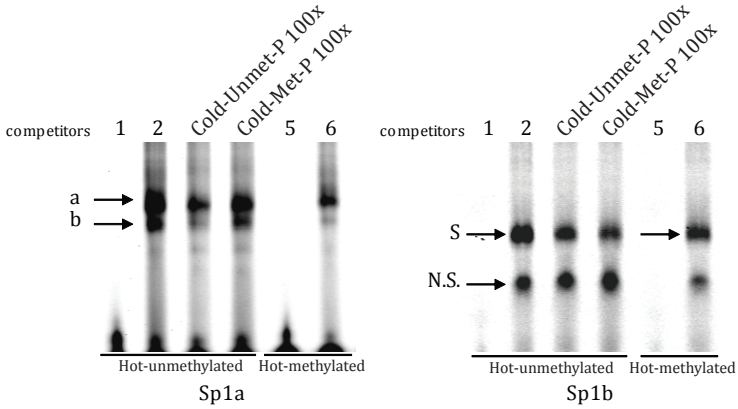


Figure 5. Interference of DNA methylation on binding of Sp1.
Competition EMSA's were performed with hot (un)methylated Sp1a and Sp1b probes and nuclear extracts (NE) of OVCAR3 cells. The specificity and methylation sensitivity of the band of interest were shown by using the cold competitors (lane 1,5: probe; 2,6: probe with NE; 3,4: probe with NE in the presence of 100-fold excess of indicated competitor).

DISCUSSION

Epigenetic aberrations, including DNA methylation and histone modifications, are well established in the development and progression of ovarian cancer (31, 32). A number of protein coding genes are overexpressed in ovarian cancer due to loss of DNA methylation, including maspin, claudin-3 (33) and claudin-4 (32). In addition, overall loss of the repressive histone mark H3K27me3 has been associated with poor prognosis in ovarian cancer (34). In this study, we set out to unravel the epigenetic marks underlying EpCAM overexpression in ovarian cancer.

In our ovarian cancer cell line panel, EpCAM expression was inversely correlated with the DNA methylation status of the promoter and part of exon 1, as reported for several other tumour types (17, 18, 19, 35). Interestingly, treatment of our EpCAM negative ovarian cancer cells with a DNA methylation inhibitor induced EpCAM expression, both on mRNA and protein level. The role of DNA methylation in silencing EpCAM has been previously published by us for the intermediate EpCAM expressing SKOV3 ovarian cancer cells, and is in line with observations in other tumour types (17, 18, 19). In normal cells (HOSE) we did not find DNA hypermethylation even though in

several ovarian cancer cell lines, including SKOV3, CaOV3 and OVCAR3, EpCAM mRNA was reported to be 3 log higher compared to HOSE cells (8). Also on protein level, HOSE cultures showed negative to negligible levels of EpCAM expression (13). This unexpected low DNA methylation level for EpCAM HOSE cells is in line with data of differentiating human embryonic stem cells, where EpCAM silencing was not associated with increased DNA methylation (36). In these cells, EpCAM silencing was associated with a reduction of active histone marks and an enhancement of repressive histone marks (36). Also in our panel of EpCAM-negative cell lines, we found the silenced EpCAM promoter to be associated with repressive marks (H3K9 or H3K27 trimethylation); while relative low levels of active histone marks (H3/H4 acetylation, H3K4 trimethylation) were observeThe epigenetic marks found in the EpCAM-negative cells indicate a closed chromatin conformation, which might explain the absence of association of any of the tested transcription factors with the EpCAM gene in H134S and A2780 cells. Out of the sixteen tested transcription factors, ten were associated with the promoter in the EpCAM expressing cells. We are the first to show association of AP2α, Ets1, Ets2, E2F2, E2F4 and STAT3 with the EpCAM gene. Of special interest are the associations found for nuclear AP2α and Ets-1, as high levels of these transcription factors have been related to poor prognosis in ovarian cancer (27, 28). Similarly, a high E2F2 to E2F4 ratio was reported to be of prognostic value for ovarian cancer-free survival (29). Also STAT3 is overexpressed in ovarian cancer compared to normal or benign ovarian tumour tissue, and its expression was significantly higher in FIGO stage III/IV compared to stages I/II (30). Although our data indicate that the relation between transcription factors and clinical parameters might partially take place via EpCAM expression regulation, the direct biological significance needs to be further established.

For the transcription factors, p53, NF-κB, Sp1 and LEF-1 (25) evidence for regulating EpCAM expression was previously demonstrated in other tumour types. Although wild-type p53 and not mutant p53 has been reported to repress EpCAM expression (24), we did not observe p53 association in EpCAM negative cells. The observed association of p53 in the EpCAM-positive, p53-mutant OVCAR3 cells (37) is in agreement with the acetylated histones associated with the promoter in these cells, as mutant p53 recruits the histone acetyl transferase p300 (38). In the presence of p300, the repressive action of NF-κB on EpCAM promoter activity was abolished (26), which might explain the association of NF-κB and acetylated histones with the EpCAM promoter in EpCAM-positive cells. Also Sp1 has previously been reported to be involved in EpCAM transcription (18). Interestingly, our observation that the CpG located at -231 within the Sp1 binding site was methylated in EpCAM-negative ovarian cell lines and unmethylated in the EpCAM-positive lines was also reported for several other types of tumours (35). Together with our *in vitro* finding that methylation of this particular CpG affects Sp1 binding, this

region is currently explored by us for targeted DNA methylation approaches (20).

Apart from DNA methylation and histone modifications, other epigenetic mechanisms, including non-coding RNAs, may be (directly and indirectly) involved in EpCAM gene regulation. In this respect, microRNA-181 has been shown to upregulate EpCAM gene expression, possibly via a positive feedback loop between miR-181 and Wnt/β-catenin signalling (39). These observations are in line with our data showing association of the Wnt-pathway transcription factor LEF1 with the active EpCAM promoter. Alternatively, several endogenous non-coding RNAs have been reported to be capable of modulating gene expression directly on the transcriptional level e.g. by inducing DNA methylation (40).

At present, EpCAM is exploited as therapeutic target in several antibody-based clinical trials. Recently, the European Medicines Agency approved the use of catumaxomab for the intraperitoneal treatment of malignant ascites (14). The oncogenic role of EpCAM broadens the interest to use EpCAM not only as an immunotarget, but also as a target for epigenetic silencing. In this respect, transient siRNA-mediated silencing of EpCAM expression has been shown to reduce the oncogenic potential of breast (5), gastric (41), hepatocellular (25) and oral squamous cell (42) carcinomas.

To silence gene expression in a more sustained way, targeted DNA methylation has been achieved by fusing a DNA methyltransferase to a DNA binding domain like zinc fingers (43). Similarly, transcription effector domains fused to zinc fingers targeting the EpCAM promoter modulated EpCAM promoter activity (44). Recently, we showed that an EpCAM specific Triple helix Forming Oligonucleotide coupled to a methyltransferase variant is able to target methylation predominantly to a specific CpG in the EpCAM promoter (20). Interestingly, targeted methylation in living cells induced dense methylation up to 380bp on both sides of the target site (45), suggesting that initial DNA methylation might serve as a trigger for DNA methylation spreading. Elucidating the regulation mechanisms of EpCAM in ovarian cancer as presented here thus opens up new possibilities to exploit EpCAM as a therapeutic target.

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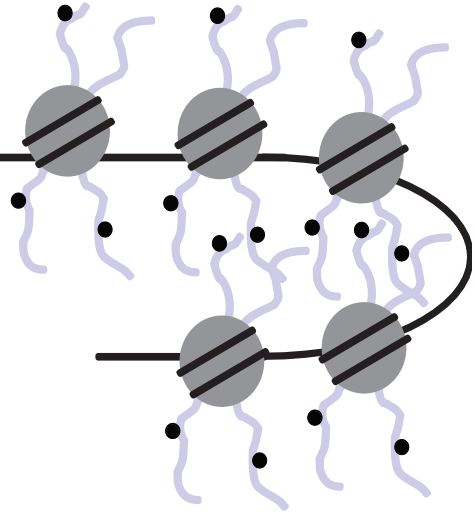
Chapter 5



Re-expression of epigenetically silenced ICAM-1 elucidates its cell-biological role in ovarian cancer cells

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Submitted for publication

ABSTRACT

Ovarian cancer is a difficult-to-treat cancer with a 5-year survival rate of only ~45 %, partially due to late diagnosis and therapy resistance. In need of new therapeutic approaches, induction of InterCellular Adhesion Molecule-1 (ICAM-1) expression might be of interest, since the expression of ICAM-1 is lower in ovarian cancer cells compared to healthy ovarian cells and positively correlated with decreased tumorigenicity. Whereas ICAM-1 expression on tumor cells is described to be of importance for adherence to immune cells, the cellular role of ICAM-1 in tumorigenicity and therapy resistance of ovarian cancer is unclear.

Here, we confirmed epigenetic silencing of the ICAM-1 gene in ovarian cancer cells, enabling re-expression from the endogenous locus. To investigate its cell-biological role, ICAM-1 expression was gene-specifically induced *in vitro* in a panel of ovarian cancer cells via retroviral transduction of Artificial Transcription Factors (ATFs). Subsequently, tumor growth and cisplatin sensitivity were evaluated with MTT assays and activation of monocytes by transduced ovarian cancer cells (with induced ICAM-1 expression) was determined by ICAM-1 protein staining of the monocytes upon co-culture.

Induction of ICAM-1 expression ranged from 2- to 228-fold on mRNA level and 1.7- to 108-fold on protein level. This resulted in a slight activation of monocytes, a decrease of ovarian cancer cell growth (up to 2-fold) and (consequently) reduced cisplatin sensitivity.

This study shows that, apart from its established role in the immune system, ICAM-1 plays a role in cell biological behavior of ovarian cancer cells. Moreover, this study shows that re-expression by ATFs represents a powerful approach for target validation of genes epigenetically silenced in cancer, like ICAM-1.

INTRODUCTION

Ovarian cancer is one of the most difficult to treat cancers with a five-year survival rate of only ~45% (in the UK (1)), which is partially due to subtle early symptoms, resulting in late diagnosis. The use of platinum based agents such as cisplatin is one of the major therapies for ovarian cancer. However, the therapeutic effects of cisplatin are insufficient because many patients either develop resistance against cisplatin or intrinsically resistant (cancer stem) cells eventually grow out (2). Thus, there is a need for new approaches and novel therapeutic targets. Immunotherapy to attract immune cells is an example of such an alternative approach that is currently explored to increase therapeutic efficacy for ovarian cancer patients (3).

In this respect, overexpression of proteins (in tumor cells) that provoke an immune response, such as InterCellular Adhesion Molecule-1 (ICAM-1) (4), might be an effective approach. Indeed, the percentage of ICAM-1 positive tumor cells was positively correlated with the amount of mononuclear infiltration in renal-cell cancer (5). In addition, for the majority of ovarian cancer cell lines and primary ovary tumors, the expression of ICAM-1 was shown to be reduced compared to healthy ovary cells, as described by Arnold *et al.* and references therein (6). In fact, a significant positive association was observed between ICAM-1 expression and survival of ovarian cancer patients (6). In line with these findings in ovarian cancer, also for other cancer types (including lymphoma, colorectal cancer and head and neck cancer) tumor growth and metastasis were lower and a better prognosis was reported for patients with ICAM-1 positive tumors compared to ICAM-1 negative tumors (7, 8, 9).

Despite these reports, suggesting a tumor suppressive role for ICAM-1, ICAM-1 expression can also negatively affect prognosis of a patient, as studies in e.g. melanoma and gastric cancer associated higher ICAM-1 expression with an increase in metastasis (10, 11). Adhesion of ICAM-1 expressing tumor cells to activated leukocytes and thereby induction of migration of tumor cells away from the primary tumor was suggested to explain the observed metastasis.

Dependent on cellular context, ICAM-1 seems to have different roles in tumorigenicity and even within the same cell type inconsistent data was reported: in one breast cancer study ICAM-1 expression on the tumor cells was correlated with reduced tumorigenicity (12), whereas in another study (13) increased tumorigenicity was shown when ICAM-1 was expressed on the breast cancer cells. This discrepancy might be explained by the differential expression of other genes that were not taken into account in these studies, showing the need of further specific investigation of single genes.

To address the biological role of one particular gene, Artificial Transcription Factors (ATFs) are a suitable tool, since they specifically modulate the expression of the target gene. ATFs consist of DNA binding domains, specifically engineered to recognize a

sequence of interest, such as Zinc Fingers (ZFs)(14), fused to transcriptional activation or repression domains, like VP64 (four copies of the viral protein VP16) or SKD (Super KRAB Domain), respectively (15). Such ATFs have previously been shown by us(16) and others(17, 18) to effectively up- or downregulate genes both *in vitro* and *in vivo*, including epigenetically silenced genes and including ICAM-1 (18).

In this study, ATFs were exploited to determine the cell-biological role of ICAM-1 in ovarian cancer. Gene-specific induction of ICAM-1 expression resulted in inhibition of ovarian cancer cell growth and (thereby) in decreased sensitivity of the ovarian cancer cells to cisplatin. This study is the first to describe an immune-response-independent cellular role of ICAM-1 in ovarian cancer.

MATERIALS AND METHODS

Cell culture

Ovarian cancer cell lines A2780, SKOV3, H134S, OVCAR3 and CaOV3 and virus producing HEK293T cells were cultured in DMEM (BioWhittaker, Walkersville, MD, USA), supplemented with 50 μg ml^{-1} gentamicin sulphate, 2 mM L-glutamine and 10% FBS. Monomac-6 cells (19) were cultured in RPMI-1640 medium (BioWhittaker), supplemented with 50 μg ml^{-1} gentamicin sulphate, 2mM L-glutamine, 10% FBS, 1mM Na-pyruvaat and 0.05 mM ß-mercapto-ethanol. All cells were cultured at 37°C under 5% CO_2. Cells were treated for 24 hrs with 20 ng ml^{-1} TNF-α or every 24 hrs with 5 μM 5-Aza-2'deoxycytidine (5-aza) and then harvested for subsequent analysis.

Retroviral transductions

Retroviral transductions were performed using a standard protocol (20). HEK293T cells were transfected using calcium phosphate with virus particle producing plasmids and 7.5 μg of pMX-IRES-GFP (empty vector), pMX-CD54-opt31 (ZF only), pMX-CD54-opt31-VP64 (ZF-VP64) or pMX-CD54-opt31-SKD (ZF-SKD), kindly provided by C.F. Barbas III (The Scripps Research institute, La Jolla, CA, USA)(18). 48 hrs after transfection, medium of the HEK293T cells was harvested and replenished. The harvested medium was supplemented with FBS and 5 μg ml^{-1} polybrene (Sigma, St Louis, MO, USA) and added to the ovarian cancer host cells. This procedure was repeated the next day. 72 hrs after the final transduction, host cells were harvested and divided for RNA isolation, protein staining, or cellular read out assays.

Co-culture monomac-6

For co-culture experiments, transduced A2780 and SKOV3 cells were replated 72 hrs after the final transduction. 24 hrs later, medium was removed and monomac-6

cells were added to obtain a 1:1 ratio. After 24 hrs of coculture, cells were harvested, stained for CD45 and ICAM-1 and analyzed by flow cytometry. In parallel, monomac-6 cells were stimulated with 2 μg ml^{-1} LPS for 4 hrs as a positive control.

qRT-PCR

RNA was isolated using a Qiagen RNeasy plus mini kit (Qiagen, Hilden, Germany). Subsequently, cDNA was synthesized with random hexamer primers using the Fermentas Revertaid cDNA synthesis kit (Fermentas, Leon-Rot, Germany). qRT-PCR was executed with 10 ng cDNA for ICAM-1 expression (Taqman gene expression assay Hs00164932_m1; Applied Biosystems, Carlsbad, CA, USA) and for GAPDH expression (primers: Fw 5'-CCACATCGCTCAGACACCAT-3', Rev 5'-GCGCCCAATACGACCAAAT-3' and probe: CGTTGACTCCGACCTTCACCTTCCC (Eurogentec, Maastricht, Netherlands)) using an ABI ViiA7™ real-time PCR system (Applied Biosystems). Results are shown as relative expression compared to GAPDH levels, using the ΔCt values method.

Flow cytometry

Cells were incubated with 100 μl of mouse-anti-ICAM-1 mAB (hu5/3-2.1 hybridoma supernatant, kindly provided by Dr. M. A. Gimbrone, Harvard Medical School, Boston, MA, USA) at 4°C for 1 hr. After washing with PBS, the cells were incubated with 100 μl of PE- or FITC-labeled rabbit-anti-mouse secondary antibody (R0439 or F0313 respectively; DAKO, Glostrup, Denmark), diluted 1:40 in 5% pooled serum in PBS at 4°C for 30 minutes. To distinguish monocytes, cells harvested after co-culture experiments were additionally incubated with 100 μl PE-labeled mouse-anti-human CD45 antibody (cat. No. 304008; Biolegend, Uithoorn, Netherlands), diluted 1:60 in 5% mouse serum in PBS. After the final wash, cells were resuspended in PBS and analyzed using a BD FACS Calibur flow cytometer (Beckton Dickenson Biosciences, San Jose, CA, USA).

Bisulfite sequencing

DNA was isolated using the Quick-gDNA™ MiniPrep kit (D3007, Zymo research via Baseclear, Leiden, Netherlands). Bisulfite conversion was performed using the EZ DNA methylation gold kit (D5006, Zymo research). ICAM-1 DNA was amplified with primers 5'-GATTTAAGTTTAGTTTGG-3' (Fw) and 5'-TCACCTAAAAACAAAACCCC-3' (Rev) (see Fig. 1a). Subsequently, the PCR product was extracted from gel (QIAquick Gel extraction kit, Qiagen) and ligated in the pCR2.1 vector (Invitrogen TA cloning kit, Invitrogen, Life technologies, Bleiswijk, the Netherlands). Clones were sequenced and subsequently analyzed using the BISMA software (http://biochem.jacobs-university.de/BDPC/BISMA/).

Pyrosequencing

To quantify methylation, five CpGs (#10-14, see Fig. 1a) were subjected to pyrosequencing. Bisulfite converted DNA was amplified using primers 5'-GGGGAAGTTGGTAGTATTTAAAAGT-3' (Fw; 5' side biotinylated) and 5'-CCTTCCCCTCCCAAACAAATACTACAATTA-3' (Rev) (Biolegio, Nijmegen, the Netherlands) using the Pyromark PCR kit (Qiagen). Subsequently, the amplification product was sequenced, using primer 5'-ATTTCCCAACTAACAAAATACCC-3' on a Pyromark Q24 machine (Qiagen).

Matinspector

Prediction of binding of transcription factors to the ICAM-1 promoter was performed using Matinspector software (Genomatix, Munich, Germany) analyzing matrices for vertebrates and general core promoter elements with default settings (Fig. 1b).

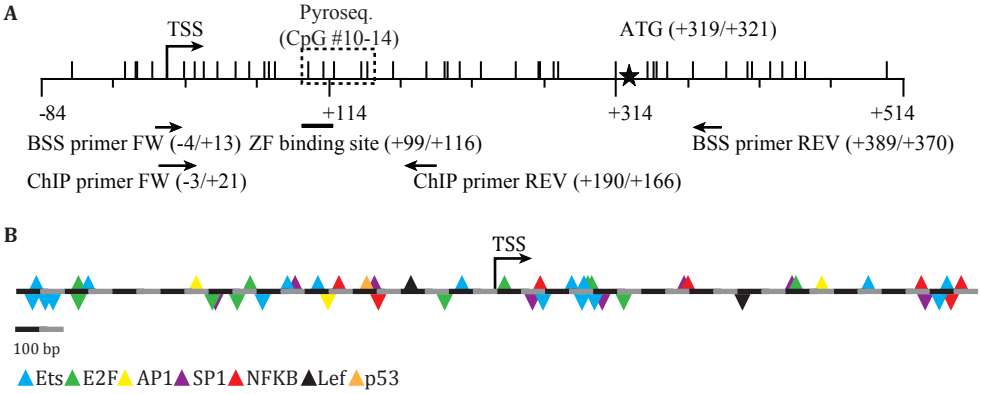


Figure 1. Schematic representation of ICAM-1 promoter around TSS.

(A) Vertical bars are CpG sites. The arrow designated with TSS indicates the position of a described transcription start site. The translation start site (ATG) is indicated with a star. All positions are in base pairs relative to the TSS. Arrows below the figure show the binding positions of the primers used for bisulfite sequencing (BSS) and ChIP. CpGs surrounded by dashed box (#10-14) were analyzed by pyrosequencing. With a horizontal line, the ZF binding site is depicted. (B) Putative binding sites of a set of transcription factors as predicted by Matinspector.

Chromatin ImmunoPrecipitation (ChIP)

Chromatin ImmunoPrecipitation (ChIP) was performed essentially as described before (21). ChIP for histone modifications was performed according to the Upstate Biotechnology (Lake Placid, NY, USA) protocol and for transcription factors, the protocol described by Weinmann and Farnham was used (22). Antibodies used were from Upstate Biotechnology (Lake Placid, NY, USA), except for mouse IgG, LEF-1(REMB6)TCF (Millipore, Amsterdam, Netherlands), NF-κBp50(NLS), E2F-4(C-20) and p53 (Santa Cruz Biotechnology, Heidelberg, Germany). qRT-PCR was performed using AbsoluteQPCR SYBRGreen-ROXMix (Abgene, Surrey, UK)

and ICAM-1 ChIP primers: 5'-ATTCAAGCTTAGCCTGGCCGGGAAA-3' (Fw) and 5'-CCCTCCGGAACAAATGCTGCAGTTA-3' (Rev) (See Fig.1a) using an ABI ViiA7™ real-time PCR system (Applied Biosystems).

MTT assay

Per cell line, equal amounts of transduced cells were replated 72 hrs after the final transduction. Twenty-four, 48, 72 and 96 hrs after plating, MTT (M2128; Sigma) was added to the cell medium and incubated for 3.5 hrs at 37°C. Subsequently, cell medium was aspirated and 200 µl DMSO was added to the wells to dissolve the crystals. Absorption was measured at 560 nm and 670 nm (Varioskan microplate spectrophotometer, Thermo Scientific, Breda, the Netherlands). 670 nm values were subtracted from 560 nm values and the values for empty vector at 24 hrs after plating were set at 1 in the graphs. To determine chemo-resistance, cisplatin was added 24 hrs after plating (concentrations indicated) and MTT assays were performed 72 hrs after cisplatin addition. For each cell type, 0 µM cisplatin was set at 100% in the graphs.

RESULTS

ICAM-1 re-expression in an ovarian cancer cell line panel

In this study, a panel of ovarian cancer cell lines differentially expressing ICAM-1 was used (Fig. 2a): A2780 and SKOV3 as ICAM-1 negative or weakly expressing ICAM-1 (mRNA, relative to GAPDH: $1.1x10^{-5} \pm 2.4x10^{-6}$, Mean Fluorescence Intensity; MFI: 1.8 $\pm$ 0.5, mRNA: $1.3x10^{-4} \pm 1.8x10^{-5}$, MFI: 7.9 $\pm$ 0.8, respectively), H134S as intermediately expressing ICAM-1 (mRNA: $1.3x10^{-3} \pm 1.8x10^{-4}$, MFI: 99.5 $\pm$ 7.2) and OVCAR3 and CaOV3 as highly positive for ICAM-1 expression on mRNA level ($3.9x10^{-3} \pm 2.7x10^{-5}$ and $7.9x10^{-3} \pm 4.3x10^{-4}$, respectively), with corresponding levels of protein expression (MFI: 481 $\pm$ 109 and MFI: 1822 $\pm$ 102, respectively).

To investigate whether ICAM-1 induction from the endogenous locus is possible in ovarian cancer cells, cells were treated with a subtoxic concentration of the DNA methylation inhibitor 5-aza. This resulted in re-expression of ICAM-1 in the ICAM-1 negative cell lines (Fig. 2b). The most pronounced effect is seen in A2780 cells (over 400-fold on mRNA level; $p<0.05$), but also in SKOV3 cells induction was observed (11 $\pm$ 3-fold; $p<0.01$). Treatment of SKOV3 cells with TNF-α, known to induce ICAM-1 expression in endothelial cells(23), resulted in 145-fold upregulation of ICAM-1 expression on mRNA level (data not shown). Although TNF-α could further induce expression of ICAM-1 in ICAM-1 highly positive OVCAR3 cells (~3-fold on protein level, data not shown), no significant upregulation was observed after treating the intermediately ICAM-1 positive H134S cells with 5-aza.

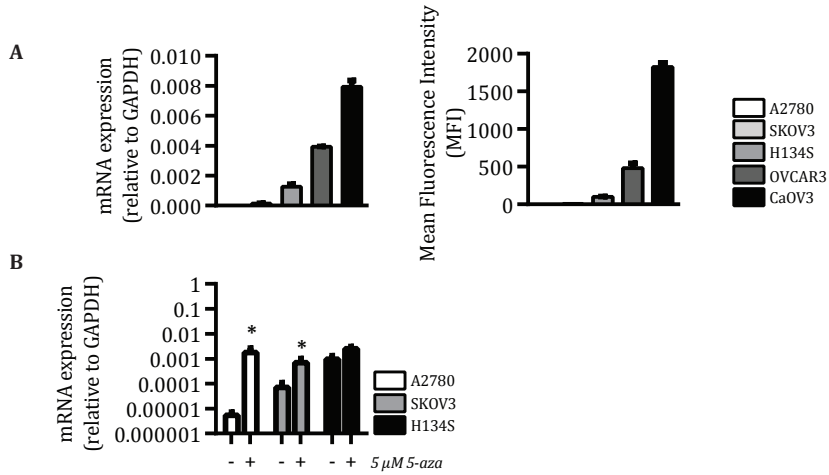


Figure 2. Pharmacologically induced re-expression of ICAM-1 in a panel of ovarian cancer cell lines. (A) Left: ICAM-1 mRNA expression relative to GAPDH of the ovarian cancer cell line panel. Error bars depict the standard deviation of the experiment performed in triplo. Right: ICAM-1 protein expression (Mean fluorescence intensity; MFI) of the ovarian cancer cell lines. The analysis was performed three times in triplicates. Error bars are the SEM of the three means of the experiments. (B) mRNA expression of ICAM-1 after daily treatment with 5μM 5-aza for 96 hrs. Experiment was performed three times in triplicate. The error bars represent the SEM of the three means of the experiments. * $p<0.05$; T-test.

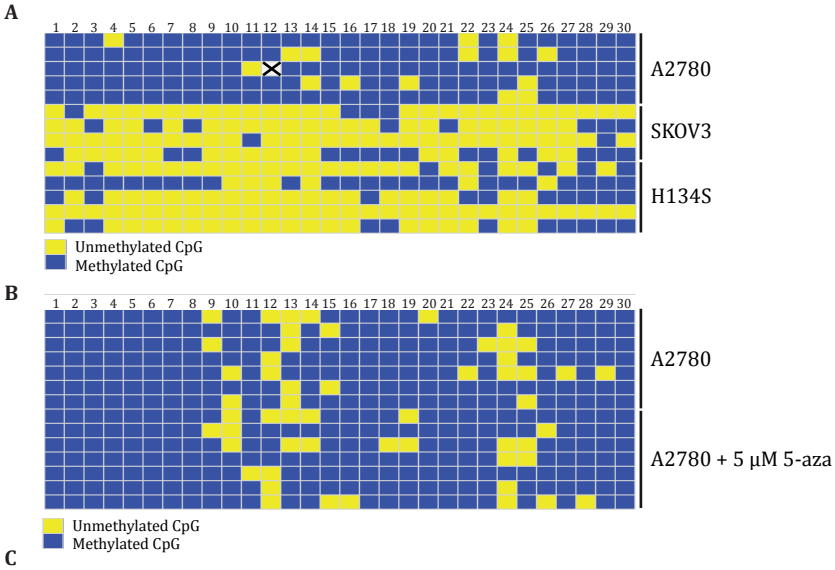
5

Epigenetic regulation of ICAM-1 expression in ovarian cancer cells in vitro

To further investigate the role of DNA methylation in the regulation of ICAM-1 expression in ovarian cancer cell lines in more detail, bisulfite sequencing of a part of the promoter region (see Figure 1 for primer positions) was performed. Hypermethylation of the investigated promoter part was observed in the ICAM-1 negative A2780 cell line (90 ± 5% of the 30 CpGs). In SKOV3 cells, weakly positive for ICAM-1 expression, and in the intermediately ICAM-1 positive cell line, H134S, the DNA of the investigated region was moderately methylated (Fig. 3a). The induction of ICAM-1 expression observed upon treatment of A2780 cells with 5-aza was not associated with hypomethylation of the ICAM-1 part surrounding the TSS, as observed by bisulfite sequencing of 5-aza treated cells (87 ± 7%) compared to untreated cells (87 ± 6%) (Fig. 3b). By pyrosequencing of 5 CpGs (#10-14), some DNA demethylation could only be detected in A2780 cells after 5-aza treatment, not in SKOV3 cells and H134S cells (Fig. 3c).

Besides DNA methylation, repressive histone modifications could underlie repression of ICAM-1 expression. Although the H3K9me3 mark is not strongly present on the ICAM-1 promoter in any of the three cell lines not, weakly or intermediately expressing ICAM-1 (A2780, SKOV3 and H134S), the repressive mark H3K27me3 was detected on the ICAM-1 promoter in all three cell lines with an average fold enrichment over IgG ranging from 17 in H134S to 331 in SKOV3. Interestingly, in contrast to the

ICAM-1 negative or weakly positive cell lines, in the moderately ICAM-1 expressing cell line H134S, the promoter was co-occupied by the active mark H3K4me3. The active marks histone H3 and H4 acetylation were detected at the ICAM-1 promoter in all cell lines, whereas H3K4me1 and H3K36me2 were not or only slightly present on the ICAM-1 promoter independent of the expression status (data not shown).



C

5-aza / CpG#	A2780		SKOV3		H134S	
	-	+	-	+	-	+
10	73.5%	66.5%	11.3%	21.4%	8.7%	12.3%
11	79.1%	66.3%	18.3%	23.6%	15.8%	17.0%
12	61.9%	56.0%	15.5%	20.0%	13.1%	15.0%
13	55.5%	51.0%	7.8%	19.7%	6.2%	8.2%
14	62.4%	59.1%	14.6%	18.9%	7.8%	10.1%

Figure 3. DNA methylation levels in ovarian cancer cell panel.
(A) Bisulfite sequencing results of part of the ICAM-1 promoter surrounding the TSS. Every square represents one CpG. Yellow squares depict unmethylated CpGs, blue squares depict methylated CpGs. (B) Bisulfite sequencing results after treating cells with 5 µM 5-aza. (C) Pyrosequencing results of 5 CpGs (see fig. 1) after treatment with 5-aza.

Transcription factor binding of the ICAM-1 promoter in ovarian cancer cell lines

To determine whether repressive epigenetic marks (DNA methylation and H3K27me3) are associated with a lack of transcription factor binding at the TSS region of the ICAM-1 promoter in the ICAM-1 negative A2780 cells compared to the ICAM-1 intermediately positive H134S cells, immunoprecipitation of several transcription factors was performed. First, the binding of certain transcription factors to the ICAM-1

promoter was predicted *in silico* (Fig. 1b). By ChIP, occupation of the ICAM-1 promoter around the TSS by NF-κB, SP1, E2F4, and p53 (predicted to bind the ICAM-1 promoter) was detected in both A2780 and H134S. The average fold enrichment over IgG was 8.6 in A2780 versus 4.4 in H134S (data not shown). In both cell lines, Lef1, also predicted to be binding the investigated part of the promoter, was not observed to bind (data not shown). Previously, none of the transcription factors described here were detected to bind the EpCAM promoter in the same IPs on these cell lines (both negative for EpCAM expression) (21). The folds of enrichment that were observed for ICAM-1 are comparable (or even higher) to those observed for EpCAM in e.g. OVCAR3 and CaOV3 which are highly EpCAM positive (21). So, despite the differences seen in epigenetic marks, the negative epigenetic marks are not associated with a lack of TF binding in this region.

Biological role of ICAM-1 in ovarian cancer

To explore the cell biological role of ICAM-1 in ovarian cancer, the expression of ICAM-1 in the ovarian cancer cell line panel was modulated by artificial transcription factors (ATFs). Depending on the effector domain fused to the ZF, both up and downregulation of ICAM-1 expression on mRNA and protein level was observed when compared to the empty vector (Fig. 4). ICAM-1 expression was significantly upregulated on mRNA level by ZF-VP64 in all five cell lines of the panel, ranging from 2.0 ± 0.2-fold ($p<0.001$) in intermediately ICAM-1 positive H134S cells to 228 ± 36-fold ($p<0.001$) in weakly ICAM-1 expressing SKOV3 cells. On protein level, the amount of upregulation ranged from 1.7 ± 0.2-fold ($p<0.01$) in the ICAM-1 positive CaOV3 cells and 1.8 ± 0.6-fold ($p<0.01$) in the intermediately ICAM-1 positive H134S cells to 108 ± 29-fold ($p<0.05$) in the weakly ICAM-1 expressing SKOV3 cells. Significant downregulation was observed on mRNA level in all cell lines, ranging from 35 ± 13% ($p<0.01$) in intermediately ICAM-1 expressing H134S to 94 ± 1% ($p<0.001$) in weakly ICAM-1 expressing SKOV3 cells. In the ICAM-1 negative cell line A2780, although downregulation seems to occur on mRNA level, the effect is the same as for the zinc finger only, and thus not relevant. Indeed, on protein level, all cell lines except for the already ICAM-1 negative cell line A2780 showed significant downregulation, ranging from 37 ± 11% ($p<0.01$) in the intermediately ICAM-1 expressing cell line H134S and 47 ± 14% ($p<0.05$) in the weakly ICAM-1 expressing cell line SKOV3 to 91 ± 2% ($p<0.001$) in the highly ICAM-1 expressing OVCAR3 cells.

To investigate the effect of induction of ICAM-1 expression by ZF-VP64 on the growth of ovarian cancer cell lines, MTT assays were performed on four consecutive days after plating equal amounts per cell line at day 0 (Fig. 5a). In the ICAM-1 negative A2780 cells as well as in the weakly ICAM-1 expressing SKOV3 cells, a reduction in proliferation was

observed in four days when transduced with ZF-VP64 compared to transduced with the empty vector (n=3). Even in the highly ICAM-1 positive OVCAR3 cells, further induction of ICAM-1 by ZF-VP64 reduced proliferation, whereas repression of ICAM-1 by ZF-SKD did not alter tumor cell growth (n=3). Effects seen in these experiments might very well be an underestimation since the cells at day 0 are cells replated 72 hrs. after the final transduction, a time-frame in which a fraction of the transduced cells likely has already died.

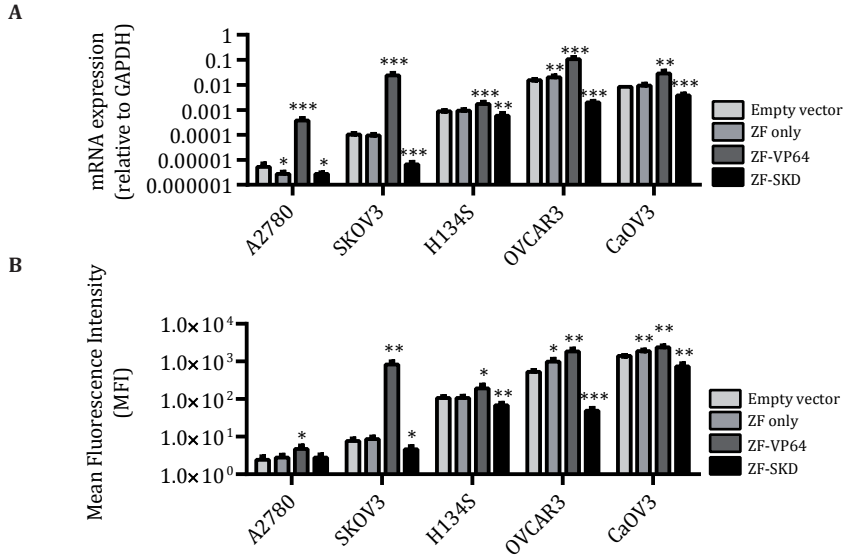


Figure 4. Bidirectional ICAM-1 expression modulation by ATFs.

Top: mRNA expression of ICAM-1 relative to GAPDH after transducing cells with ATFs. The error bars depict SD of the experiment performed in triplo. Bottom: Protein expression (MFI) of ICAM-1 after transducing cells with ATFs. The error bars depict the SEM of the means of the three experiments. * p<0.05, ** p<0.01, *** p<0.001, T-test compared to empty vector.

To study the role of ICAM-1 expression in (partially) controlling cisplatin sensitivity of ovarian cancer cells, cells transduced with ATFs were incubated with different doses of cisplatin for 72 hrs. and proliferation was analyzed by MTT assays (Fig. 5b). At a concentration of 10 μM cisplatin, 86 ± 33% of A2780 cells transduced with ZF-VP64 survived, compared to 26 ± 7% for ZF-SKD and 16 ± 11% for the empty vector after 4 days compared to day 1. For SKOV3, this was 82 ± 27% , 25 ± 3% and 33 ± 11%, respectively. In the highly ICAM-1 positive cell lines OVCAR3, the effects of further increase of ICAM-1 expression on cisplatin sensitivity were less clear, while in CaOV3 no changes in cisplatin sensitivity were seen upon transduction with viral constructs.

In order to investigate whether ICAM-1 induction on ovarian cancer cells leads to activation of co-cultured monocytes, ICAM-1 expression on Monomac-6 cells was measured after 24 hrs of co-culture with ATF-transduced ovarian cancer cells (Fig.

5c). Whereas LPS could increase ICAM-1 expression on Monomac-6 cells 5.0 ± 0.8-fold ($p<0.01$), A2780 transduced with ZF-VP64 increased ICAM-1 expression on Monomac-6 cells only 1.3 ± 0.1-fold ($p<0.05$) compared to untreated A2780 cells. No significant upregulation of ICAM-1 expression on Monomac-6 cells was observed when co-cultured with ZF-VP64 transduced SKOV3 cells.

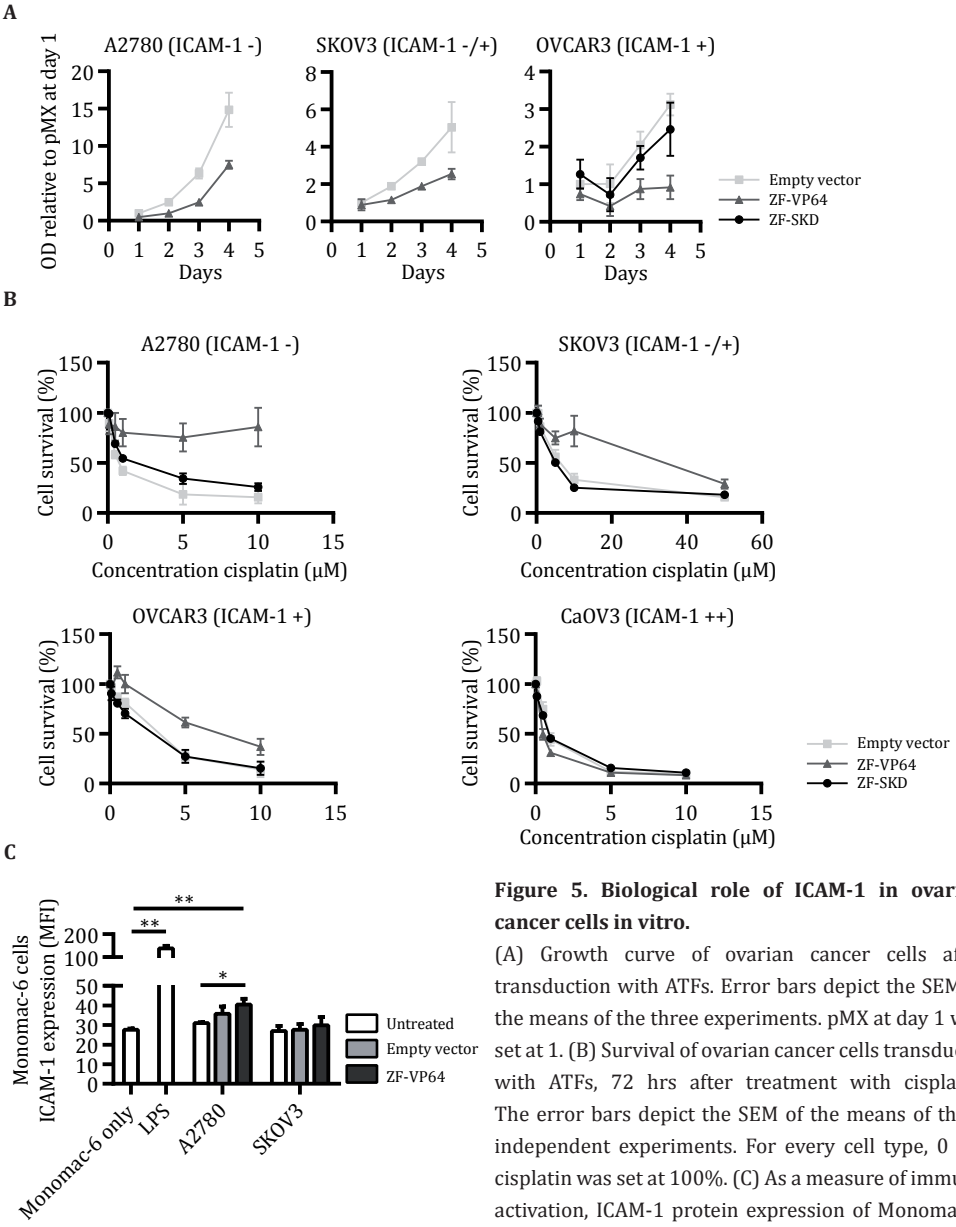


Figure 5. Biological role of ICAM-1 in ovarian cancer cells in vitro.

(A) Growth curve of ovarian cancer cells after transduction with ATFs. Error bars depict the SEM of the means of the three experiments. pMX at day 1 was set at 1. (B) Survival of ovarian cancer cells transduced with ATFs, 72 hrs after treatment with cisplatin. The error bars depict the SEM of the means of three independent experiments. For every cell type, 0 μM cisplatin was set at 100%. (C) As a measure of immune activation, ICAM-1 protein expression of Monomac-6 cells is shown after 24 hrs of co-culture with untreated or ATF-transduced ovarian cancer cells.

DISCUSSION

In this study we show, apart from the known tumor suppressive role of ICAM-1 via activation of monocytes, which was described before (24, 25), that induction of ICAM-1 expression in ovarian cancer cells reduces tumor cell proliferation and cisplatin sensitivity *in vitro*. To the best of our knowledge, this is the first study on the functional cell-biological role of ICAM-1 in ovarian cancer.

The beneficial role of ICAM-1 in ovarian cancer, shown in the present study by using ATFs *in vitro*, is in line with results obtained upon transfecting ICAM-1 cDNA into various cancer cells, where overexpression of the gene was associated with decreased tumorigenicity *in vivo* (26, 27, 28, 29, 30, 31). In more detail, lower proliferation rates were observed when cancer cells (other than ovarian) were transfected with ICAM-1 cDNA and implanted into animal models (28, 29, 31). The reduced growth *in vivo* has been ascribed to increased activation of immune response by the cells upon ICAM-1 expression. Indeed, when the effect on growth of the cancer cells expressing ICAM-1 cDNA was studied *in vitro*, no tumor growth inhibition was observed, for colon and liver cancer cells (29, 31). The different outcome between cDNA transfection in colon or liver cancer cells and ATF induced ICAM-1 expression in our experiments might be explained by the difference in tumor types (colorectal/liver cancer versus ovarian cancer). Actually, multiple examples are known of genes displaying cell-context dependent seemingly controversial roles, like EpCAM (32) or Maspin (33). In this respect, in breast cancer cells, silencing of ICAM-1 expression by siRNA resulted in decreased tumorigenicity *in vitro* (34, 35).

Importantly, in the cDNA studies in colorectal or liver cancer only one isoform of ICAM-1 is expressed, whereas the ATFs used in the present study ensure expression of all isoforms in their natural ratios, which was previously shown to be of importance for the functionality of other genes (36). For ICAM-1, thus far at least seven isoforms have been described (37). Thus, our study serves as a good example of the advantage of using ATFs to investigate the role of a gene of interest.

Next, we were interested to see whether induction of ICAM-1 expression has a direct effect on cisplatin resistance. In literature, cisplatin treatment is reported to increase ICAM-1 expression (38). This might be caused by DNA demethylation, since the promoter of the ICAM-1 gene is one of the promoters that were found hypomethylated in a cisplatin resistant ovarian cancer cell line compared to its cisplatin-sensitive parental line (39). Thus, either cisplatin induces ICAM-1 expression (through DNA demethylation or indirect) or cisplatin treatment selects for high ICAM-1 expressing cells by killing the ICAM-1 low expressing cells. By means of ATF induced ICAM-1 expression, we show that it is likely that cisplatin treatment selects for ICAM-1 positive cells, since these are less sensitive to the treatment. In line with this, cisplatin induced upregulation of multiple

TNF-α target genes was prevented by a TNF-α antagonist, but not the expression of ICAM-1 (40). Whether the observed reduction in cisplatin sensitivity upon ATF induced ICAM-1 expression in ovarian cancer cells is due to the reduction in cell growth, that was observed as well, or an alternative intracellular protection pathway needs further investigation.

Previous studies showed that silencing of ICAM-1 in cancer cells, other than ovarian cancer, is likely caused by epigenetic parameters (41, 42). However, in contrast to genetic mutations, epigenetic mutations are reversible, even by environmental triggers (43), indicating that re-expression from the endogenous locus remains possible. Indeed, like Arnold *et al.*(6), we were able to re-express ICAM-1 in ovarian cancer cells using a DNA methylation inhibitor. Nevertheless, we only show a slight decrease in methylation level in 5 CpGs in the TSS region of the ICAM-1 promoter in A2780 cells upon 5-aza treatment. It could be that methylation levels of the promoter did decrease more further upstream. However, it seems more likely that the re-expression of ICAM-1 is caused by a secondary effect. In line with this, it was shown before that despite the fact that no DNA methylation was present at the ICAM-1 promoter in tumor endothelial cells, increased expression could be observed upon treatment with 5-aza (41).

Epigenetic changes are known to play a role in carcinogenesis, also in ovarian cancer (44). As many tumor suppressor genes are now known to be epigenetically silenced, epigenetic drugs are successfully tested in clinic for various cancer types (45) (and FDA approved for hematological malignancies). Indeed, the inhibitors of DNA methylation and histone deacetylation are known to affect the expression of genes throughout the genome. However, even genes involved in metastasis are upregulated after treating cells with epigenetic drugs (46).

In contrast to epigenetic drugs, the ATFs used in this study are engineered to be gene-specific and thus uniquely suited to address questions on the biological role of a gene and in specific questions on cause or consequence of up- (or down-) regulation of any gene that is not genetically mutated. ATFs show the direct consequences of targeted gene expression modulation of the gene of interest on various biologically relevant processes, whereas current studies (e.g. transcriptomics studies) mainly base their associations on circumstantial evidence. In conclusion, this study shows the potency of ATFs in validating potential therapeutic targets, thereby fully exploiting the reversibility of aberrant epigenetics in cancer

NOTE ADDED IN PROOF

Upon cell line authentication it appeared that the H134S cell line used in this study was not clearly recognizable. The profile of the H134S cell line was best comparable to HeLa cervical cancer cells. Therefore, results obtained with this cell line should be

interpreted with caution.

ACKNOWLEDGEMENTS

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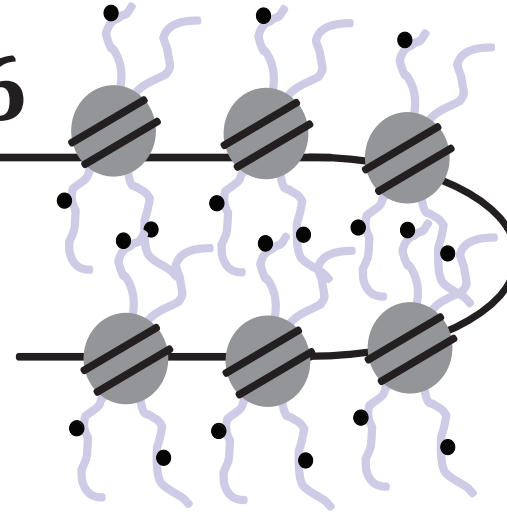
Chapter 6



Targeting putative DNA demethylases and known histone modifying enzymes specifically to endogenous genes

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Manuscript in preparation

ABSTRACT

The role of eppigenetics in the onset and progression of a variety of diseases becomes more and more evident. As an example, tumor suppressor genes are known to be aberrantly silenced in association with epigenetic marks, including DNA hypermethylation and histone modifications. Such silenced genes can be re-expressed by epigenetic drugs, but this approach has genome-wide (side) effects.

In this study, fusions of gene-specific DNA binding domains (engineered zinc fingers) and epigenetic enzymes were expressed in to target epigenetically silenced target genes for re-expression. Subsequently their effect on the level of molecular epigenetic marks at the target gene and on target gene expression was assessed.

No significant effects were observed upon targeting putative DNA demethylases, nor upon targeting the catalytic domains of UTX, an H3K27 demethylase, or p300, a histone acetyltransferase. The epigenetically silenced genes could be re-expressed by zinc fingers fused to VP64, opening up new ways to more permanently upregulate silenced genes.

INTRODUCTION

The contribution of epigenetic mechanisms in development of many diseases becomes more and more evident (1). For example, it has been shown that epigenetic silencing of tumor suppressor genes can be causative in cancer (2, 3). In fact, DNA methylation is currently used as a diagnostic/prognostic marker (4, 5, 6). DNA methylation, especially around the transcription start site or exon 1 is of a gene, is associated with repression of gene expression (7)(7, 8, 9), whereas posttranslational histone tail modifications have been associated with active or repressed genes, depending on the type, quantity and location of the modification (7, 10).

Epigenetically silenced genes do not generally have genetic mutations (11) allowing alternative therapeutic approaches. Furthermore, although both DNA methylation and histone methylation were thought to be very stable, it is now known that these epigenetic marks are reversible (12, 13). Indeed, re-expression of epigenetically silenced genes is possible, as reported for many genes. Interestingly, epigenetically silenced genes can also be gene-specifically re-expressed by using engineerable DNA binding domains (Zinc Fingers; ZFs) fused to activation domains (VP64; four copies of the viral protein VP16): Artificial Transcription Factors (ATFs) (14, 15). However, this interesting approach is likely to be transient, as VP64 functions through recruitment of activators and epigenetic marks silencing the gene are not directly affected (16).

Since DNA methylation is strongly associated with repressed genes, DNA demethylation might facilitate activation of genes. In this respect, while enzymes removing certain histone marks (including methylation) have been well identified in recent decades, the mechanisms of DNA demethylation are currently largely unknown (17, 18). Although passive DNA demethylation is commonly acknowledged in mammalian cells, being caused by the lack of inheritance of DNA methylation marks upon cell division, active DNA demethylation was not generally accepted until evidence started to accumulate (as reviewed in (18)).

Several proteins and a variety of mechanisms have been proposed to be involved in the mammalian active DNA demethylation process, as extensively reviewed (17, 18). Two of the proposed proteins are Activation Induced Deaminase (AID) and Apobec1, which were suggested to cause DNA demethylation via deamination of the methylated cytosine into a thymine base (19). The resulting T/G mismatch might then be recognized by the mismatch repair system and the thymine will be exchanged for a cytosine base. Another described mechanism for active DNA demethylation is nucleotide excision repair, in which Gadd45α was reported to be involved (20). A mechanism proven to play a role in DNA demethylation is conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), a likely intermediate in active DNA demethylation (21). This process was quite recently detected to be executed by the ten-eleven-translocase family of proteins (Tet1,

Tet2 and Tet3) (22). The formation of 5hmC is suggested to be an intermediate that can be changed into an unmethylated cytosine through several mechanisms (23, 24). 5hmC might by itself also have a function with respect to gene expression, although this is not entirely clear at present (24). Interestingly, targeting Tet1 to a predetermined site by fusion to Gal4 caused repression of the reporter gene (25) and promoter 5hmC was associated with repression of gene expression in another study as well (26). On the other hand, genome-wide hmC sequencing in mouse ES cells showed that the mark is present mainly on repressed genes, but also on active genes. Furthermore, the mark is primarily enriched at the transcription start site but also within the gene body (25).

In addition to DNA hypomethylation, several histone modifications likely underlie the expression of active genes. Also, targeted induction of positive histone modifications or removal of repressive histone modifications resulted in gene expression modulation (27). For example, acetylation of histone tail residues is commonly seen at active genes (28, 29). This acetylation is performed by histone acetyltransferases (HATs) such as p300 (30). Other histone marks, associated with repressed genes, can be removed to facilitate expression. An example of this is the action of Ubiquitously transcribed tetratricopeptide repeat, X chromosome (UTX), which demethylates H3K27me3, a mark associated with repressed genes (31, 32). H3K27me3 is also often present at tumor suppressor genes, even when they are expressed. This is because H3K4me3 is also present, allowing the gene to be expressed. This bivalent chromatin pattern, however, seems to predispose such genes to aberrant DNA hypermethylation in cancer (33, 34). In those cases, demethylating H3K27 might relieve this repression.

Identification of effects of before-mentioned putative DNA demethylases and other epigenetic enzymes was mainly performed by overexpression or knocking out the specific gene. Although these widely used systems can indeed give indications on the functions of the proteins, observed effects might be secondary. A more direct tool to get indications of answers on fundamental epigenetic questions, like which protein is truly responsible for active DNA demethylation, is by exploiting Epigenetic Editing (27). Epigenetic Editing, gene-specific rewriting of epigenetic marks, is obtained by the fusion of (candidate) epigenetic enzymes to gene-specific DNA binding domains such as ZFs.

In this study, an attempt was made to identify DNA demethylases and to investigate whether the known effect of certain histone modifying enzymes can be exploited by targeting the enzymes to predetermined endogenous sites. Gene-specific engineered ZFs were used that recognize 18 basepair sequences in the promoters of Epithelial Cell Adhesion Molecule (EpCAM) or InterCellular Adhesion Molecule-1 (ICAM-1). These endogenous, epigenetically silenced model genes have previously been re-expressed by targeting ZFs fused to a transient activation domain, VP64 (four copies of the viral protein VP16) (35, 36). In this study, Gadd45α, AID, Apobec1 and truncated versions of

Tet1, Tet3, p300 and UTX (including the catalytic domains) were fused to the ZFs. After expression of these epigenetic editors, both levels of molecular epigenetic marks and target gene expression were analyzed.

MATERIALS AND METHODS

Plasmids

Gadd45α and p300 CD (aa 1066-1707) were amplified from human cDNA using forward and reverse primers comprising AscI and PacI restriction sites in their 5' end, respectively. The forward primer amplifying p300 CD also comprised a MluI restriction site to facilitate further cloning. The amplification product of Gadd45α was inserted into pMX-Up2-IRES-GFP (36), using AscI and PacI restriction enzymes followed by three-point ligation. Murine AID, Apobec1, Tet1 CD (aa 1367-2040) and Tet3 CD (aa 697-1669) were amplified from plasmids kindly provided by dr. G.L. Xu (Shanghai Institutes for Biological Sciences, Shanghai, China) and inserted in the pMX backbone using MluI and PacI.

UTX CD (aa 401-1401) was amplified from plasmid pGvH0064 (37) kindly provided by G. van Haaften (NKI-AVL, Amsterdam, the Netherlands). In all constructs, the Up2 zinc finger (recognizing the EpCAM promoter) was replaced with the CD54 zinc finger (38) (recognizing the ICAM-1 promoter; kindly provided by C.F. Barbas III, the Scripps Institute, La Jolla, CA, USA) using the SfiI restriction enzyme to obtain CD54 fusion constructs. All PCR-cloned constructs were verified by DNA sequencing.

Cell culture

The packaging human embryonic kidney cell line (HEK293T) and human ovarian cancer cell lines (A2780, H134S and Skov3) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, BioWhittaker), supplemented with 10% Fetal Bovine Serum, 2 mM L-glutamine and 50 μg/ml gentamicin sulfate. Cells were cultured at 37°C in a humidified 5% CO_2–containing atmosphere.

Retroviral transductions

HEK293T cells were transfected with retroviral vectors encoding the ZF fusion constructs, together with the plasmids needed to produce retroviral particles, as described before (35). Virus-particle containing supernatant of the transfected cells was used to infect the host cells (A2780, H134S and Skov3) and the process was repeated after 24 hrs. Transduced cells were harvested 72 hrs after the last infection for western blot, flow cytometry analysis, qRT-PCR, bisulfite- or pyrosequencing or ChIP.

Immunoprecipitation and western blotting

Transduced cells were lysed in RIPA buffer (25 mM Tris-HCl, pH 7.6; 150 mM NaCl; 1% NP-40; 1% sodium deoxycholate; 0.1% SDS (Thermo Scientific, Waltham, MA, USA)) and centrifuged. Protein A magnetic beads (Dynabeads Protein A, Invitrogen, Life technologies, Bleiswijk, the Netherlands) were incubated with a rabbit polyclonal HA tag antibody (Novus Biologicals via Bio-Connect, Huissen, the Netherlands) at room temperature for 30 min. Supernatant of cell lysates was added and the samples were rotated at 4°C O/N. Immunoprecipitates were collected and washed four times with RIPA buffer.

Subsequently, western blotting was performed following standard procedures. Expression of the ZF fusion constructs was detected by incubating the membrane with a mouse monoclonal anti-HA tag antibody (COVANCE, Rotterdam, the Netherlands) at 4°C O/N, followed by goat anti-mouse IgG conjugated with alkaline phosphatase (DAKO, Glostrup, Denmark). Visualization was performed using BCIP/NBT substrate.

RT-PCR

Total RNA was extracted from the transduced cells using an RNeasy Miniprep Kit (Qiagen, Hilden, Germany) according to the recommendations of the manufacturer. cDNA was obtained using RevertAid Reverse Transcriptase and random hexamer primers (Fermentas, Leon-Rot, Germany). p300 containing constructs were amplified with a forward primer annealing within the p300 sequence (5' CTGCTGGATTCGTCTGTGAT 3') and a reverse primer annealing at the HA-tag sequence (5' ACGTCGTACGGGTAGTTAAT 3'). UTX containing constructs were amplified with a forward primer annealing within the UTX sequence (5' GGAAGTTGCAGCTACATGAG 3') and the same HA-tag reverse primer. Products were analyzed on an agarose gel.

qRT-PCR

For ICAM-1 or EpCAM mRNA analysis, qRT-PCR was executed on an AB ViiA7 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) with 10 ng of cDNA, using Taqman gene expression assays for ICAM-1 (Hs00164932_m1) and EpCAM (Hs00158980_m1; both Applied Biosystems). As internal control, RNA levels of GAPDH were measured using primers: Fw 5'-CCACATCGCTCAGACACCAT-3', Rev 5'-GCGCCCAATACGACCAAAT-3' and probe: CGTTGACTCCGACCTTCACCTTCCC (Eurogentec, Maastricht, Netherlands). Data was analyzed using the comparative cycle threshold method (delta Ct). Statistic significance was assessed using paired T-tests.

Flow cytometry

Harvested cells were stained for ICAM-1 or EpCAM protein expression using

mouse-anti-ICAM-1 hybridoma supernatant of hu5/3-2.1, kindly provided by Dr. M. A. Gimbrone (Harvard Medical School, Boston, MA, USA) or mouse-anti-EpCAM Moc31 hybridoma supernatant, respectively. Subsequently, cells were incubated with RaM-$F(ab)_2$-PE (DAKO). Fluorescence was measured on a BD FACS Calibur flow cytometer (Beckton Dickenson Biosciences, San Jose, CA). The living, GFP positive cells were gated to calculate the percentage of living GFP positive cells and the Mean Fluorescene Intensity (MFI) of this population for protein levels of ICAM-1 or EpCAM. Statistic significance was assessed using paired T-tests.

Bisulfite sequencing and pyrosequencing

Genomic DNA was extracted from transduced cells using the Quick-gDNA™ MiniPrep kit (D3007, Zymo Research via Baseclear, Leiden, Netherlands) and bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo Research) following manufacturers' recommendations. For ICAM-1 bisulfite sequencing, PCR products (Fig. 1a) were extracted from gel using the Qiaquick gel extraction kit (Qiagen) and cloned into the pCR2.1-TOPO vector (Invitrogen). Individual clones were send for sequencing (Baseclear).

For pyrosequencing of the ICAM-1 (Fig. 1a) or EpCAM promoter (Fig. 1b), bisulfite converted DNA was amplified by PCR. Pyrosequencing was performed on the Pyromark Q24 MD pyrosequencer (Qiagen) according to the manufacturers' guidelines. Percentage of methylation for each CpG analyzed was determined using Pyromark Q24 Software (Qiagen). Statistical significance was determined using paired T-tests versus untreated cells.

Chromatin immunoprecipitation (ChIP)

For ChIP, cells were fixated using 1% formaldehyde and subsequently sonicated to shear the DNA. Dynabeads (Invitrogen) were loaded with 5 µg of the antibodies as indicated at the figures (rIgG, H3/H4Ac and H3K27me3 from Millipore, Amsterdam, the Netherlands and H3Core from Abcam, Cambridge, United Kingdom), added to the sheared DNA and incubated overnight. Beads were washed and the DNA/protein complexes were eluted from the beads. Protein and RNA were removed and the DNA was purified and used for PCR, primer positions are indicated in Fig. 1a and 1b.

RESULTS

Expression of ZF fusion constructs

To confirm expression of the ZF fusion constructs, immunoprecipitation was performed, followed by western blotting using antibodies detecting the HA-tag (Fig.

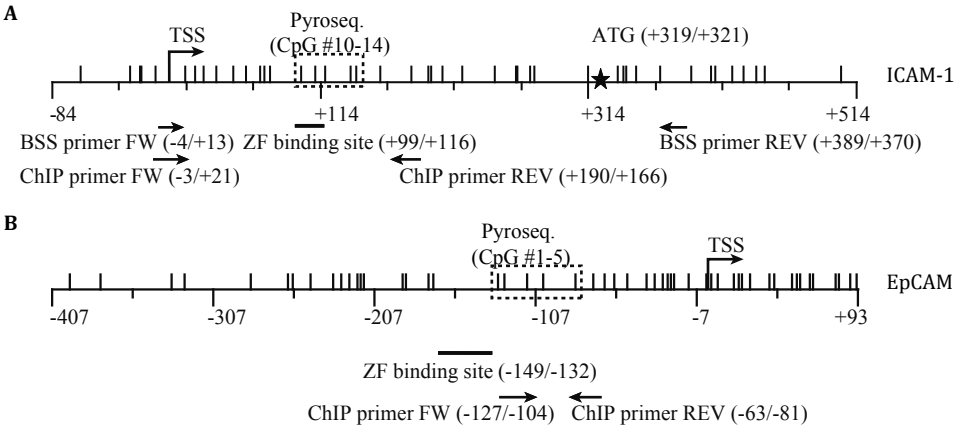

Figure 1. Promoters of model genes

Schematic representation of part of the ICAM-1 (a) and EpCAM (b) promoter. Indicated are the zinc finger binding sites, the primers used for bisulfite sequencing and/or ChIP and the CpGs analyzed by pyrosequencing.

2a). All constructs comprising putative DNA demethylases were detected at the right size. Up2 ZF containing proteins show a little higher molecular weight than the CD54 ZF containing proteins, explained by the difference in construction (38, 39). Only, a faint band was seen for Up2-Tet3, whereas no band was detected for CD54-Tet3. Also the control constructs, ZFs without effector domain or ZFs fused to VP64 were detected. The band for Up2-VP64 shows a low intensity, which is in line with the toxicity of the treatment (36). Also fusion proteins containing AID or the CDs of the Tet-proteins resulted in low intensity bands, but here no toxicity was observed. ZFs fused to p300 CD or UTX CD were not detected by western blot, but RT-PCR did show expression of these constructs in the transduced host cells (Fig. 2b).

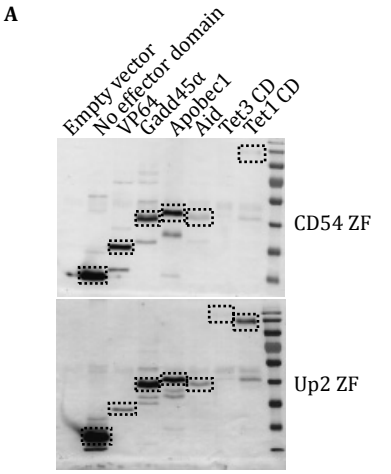

Figure 2. Expression of zinc finger fusion constructs

In this figure, detection of the expression of the constructed zinc finger fusion proteins is shown. (A) α-HA-tag immunoprecipitation followed by western blot was performed for fusion proteins containing putative DNA demethylases, targeting the ICAM-1 (top) and EpCAM (bottom) gene in A2780 cells. Correct bands are surrounded by dashed squares. (B) RT-PCR to detect expression of ZF fusions containing p300 in A2780 cells, Up2-UTX in H134S cells and CD54-UTX in Skov3 cells.

Gene-specific targeting of putative DNA demethylating enzymes: gene expression

To investigate whether targeted putative DNA demethylases were able to induce gene expression, target gene expression was assessed of two model genes in three different ovarian cancer cell lines (A2780, H134S and Skov3). In A2780 cells, negative for ICAM-1 and EpCAM expression, fusions of the two different ZFs (targeting ICAM-1 or EpCAM) to VP64 both induced gene expression on mRNA level compared to expression of the ZF only (Fig. 3a and 3b). ICAM-1 expression was induced 495 ± 376-fold ($p<0.05$) and EpCAM expression 18 ± 11-fold in the surviving cells (not significant). As described by us before (36), the Up2-VP64 fusion protein was highly toxic to the cancer cells used. Because of the small amount of cells left after treatment with Up2-VP64, the expression of EpCAM on protein level could not be determined upon expression of this construct. None of the putative DNA demethylases showed significant induction of target gene expression on mRNA level upon expression of the ZF fusion proteins in A2780 cells (Fig. 3a and 3b), nor in H134S or Skov3 cells (data not shown). On protein level, no significant expression differences were observed upon expression of the ZF-DNA demethylase fusion proteins in A2780 cells (Fig. 3c and 3d) or H134S or Skov3 cells (data not shown).

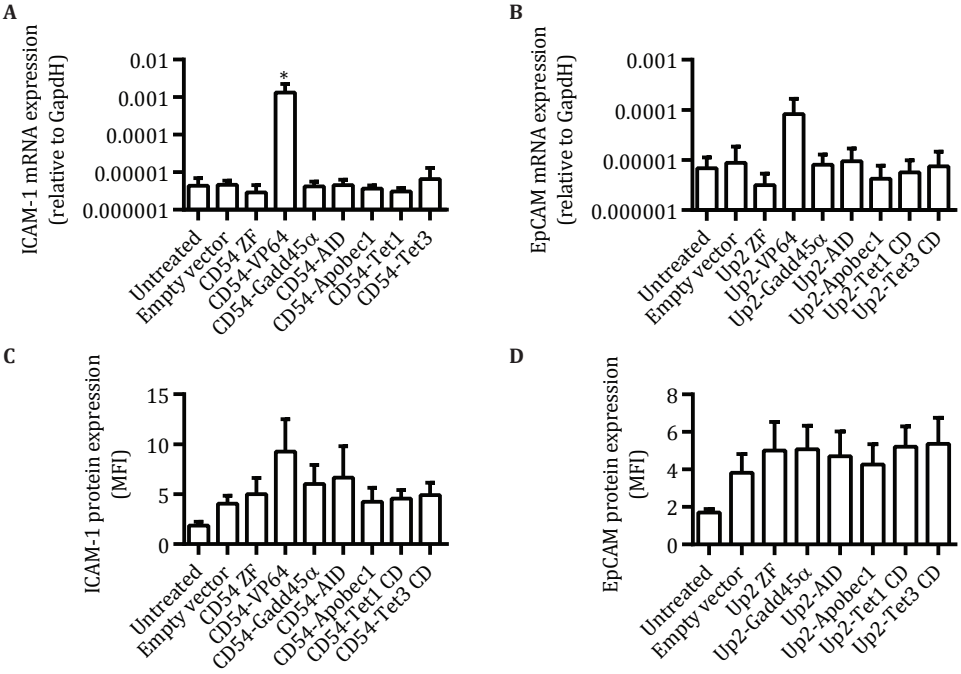

Figure 3. Target gene expression upon targeting putative DNA demethylases

Mean expression of ICAM-1 (A and C) and EpCAM (B and D) in A2780 cells after expressing fusion proteins comprising ZFs and putative DNA demethylases. Expression was analyzed on mRNA level (A and B) and protein level (C and D). MFI is mean fluorescence intensity of gated cells. Error bars represent the standard error of the mean. * $p<0.05$, paired T-test.

Gene-specific targeting of putative DNA demethylating enzymes: DNA methylation levels

Despite the fact that no changes in target gene expression were observed upon targeting the putative DNA demethylating enzymes, it might be that an effect on DNA methylation did occur. To obtain insights in which CpGs are worthwhile to analyze for quantification through pyrosequencing, first bisulfite sequencing of the ICAM-1 promoter was performed upon expression of the CD54 ZF alone or fused to VP64 or Tet1 (Fig 4a). As expected, the CpGs in the ZF binding site and those potentially affected by the effector domain (regarding the orientation of the protein) seemed most affected. Therefore, CpG #10-14 (see also Fig. 1a) were investigated in more detail by pyrosequencing to quantitatively assess the amount of DNA methylation present at these specific CpGs.

Cells treated with the CD54 ZF without effector domain and CD54-VP64 fusion protein both showed significant reduction in the percentage of DNA methylation on almost all investigated CpGs in comparison to untreated cells (For CD54 ZF, CpG #10: 29 $\pm$ 10 %, $p<0.001$; CpG #11: 27 $\pm$ 10%, $p<0.01$; CpG #12: 6 $\pm$ 4%, $p<0.05$; CpG #13: 6 $\pm$ 3%, $p<0.01$; for CD54-VP64, CpG #10: 13 $\pm$ 3%, $p<0.001$; CpG #11: 14 $\pm$ 7%, $p<0.01$; CpG #13: 8 $\pm$ 4%, $p<0.01$; CpG #14: 6 $\pm$ 5%, $p<0.05$) (Fig. 4b). Interestingly, expression of CD54-Tet1 CD and CD54-Tet3 CD also resulted in significant reduction in the percentage of DNA methylation of CpG #10 (6 $\pm$ 5% and 4 $\pm$ 3%, respectively; both $p<0.05$), although the degree of DNA demethylation is comparable to the other domains.

Analyzing CpG methylation in the EpCAM promoter was more challenging, likely due to its high CG content. As there is no CpG in the ZF binding site, CpGs directly downstream were analyzed (see Fig. 1b). CpG 1 and 2 were relatively well analyzable, whereas CpG 3 and 4 resulted in more failure of determination, especially for Up2-VP64, Up2-Gadd45α and Up2-Apobec1. Methylation levels of CpG 5 resulted in failed determination in almost all cases (data not shown). No significant differences in methylation levels was observed in the CpGs in the EpCAM promoter analyzed at least three times upon expression of the Up2 fusion proteins (Fig. 4c).

Gene-specific targeting of histone modifying enzymes: gene expression

Towards combining targeted DNA demethylases with targeted histone modifying enzymes to obtain more efficient and/or prolonged effects, the catalytic domains of a HAT (p300) or an H3K27 demethylase (UTX) were fused to the ICAM and EpCAM ZF. These specific enzymes are known to change epigenetic marks in a way which might facilitate induction of gene expression. In A2780, negative for ICAM-1 expression, again CD54-VP64 increased ICAM-1 gene expression (average 157-fold on mRNA level and 1.7-fold on protein level), but CD54-p300 did not (Fig. 5a). In Skov3, weakly expressing

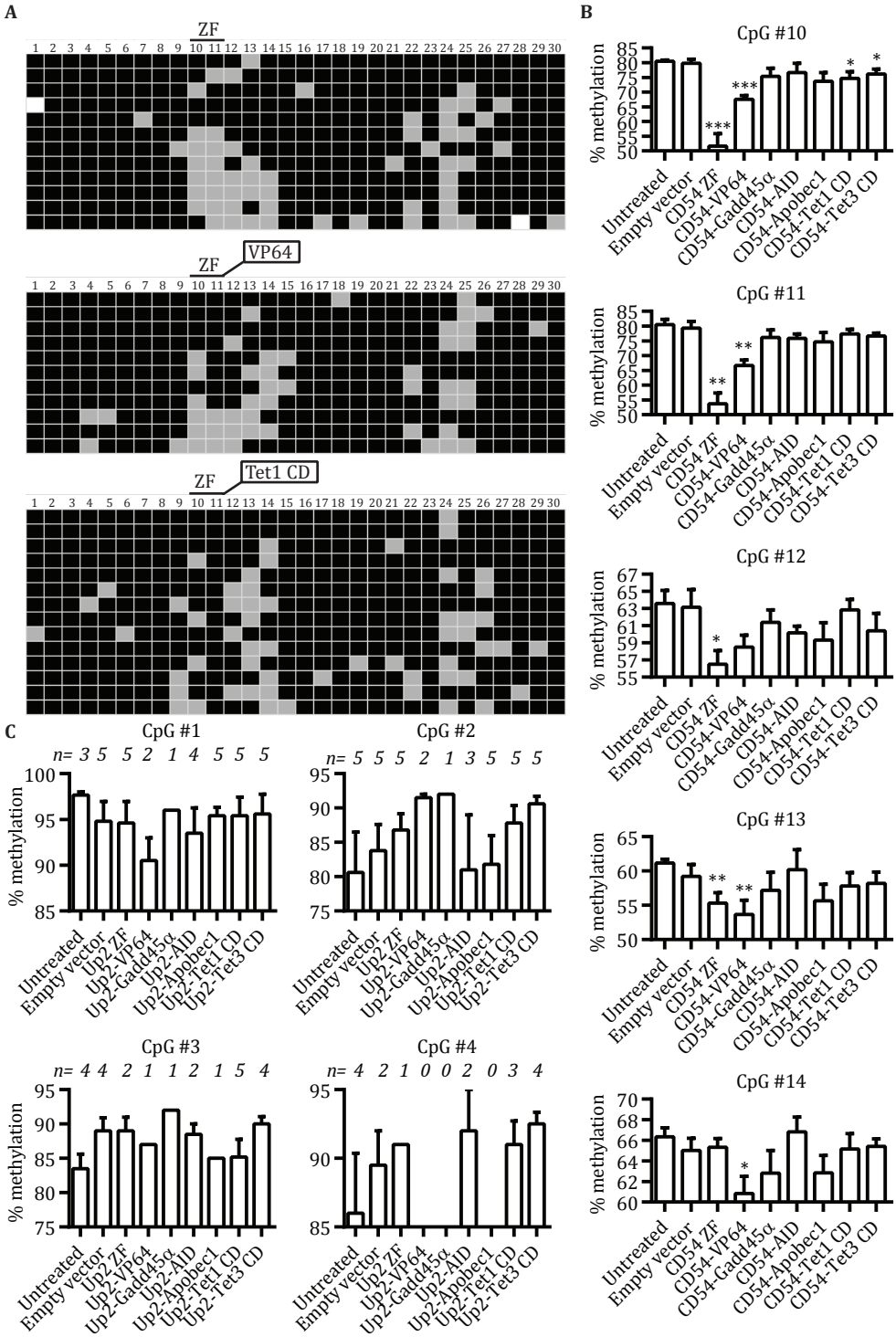
A
ZF
ZF
VP64
ZF
Tet1 CD
B
CpG #10
CpG #11
CpG #12
CpG #13
CpG #14
% methylation
Untreated
Empty vector
CD54 ZF
CD54-VP64
CD54-Gadd45α
CD54-AID
CD54-Apobec1
CD54-Tet1 CD
CD54-Tet3 CD
C
CpG #1
n= 3 5 5 2 1 4 5 5 5
CpG #2
n= 5 5 5 2 1 3 5 5 5
CpG #3
n= 4 4 2 1 1 2 1 5 4
CpG #4
n= 4 2 1 0 0 2 0 3 4
Up2 ZF
Up2-VP64
Up2-Gadd45α
Up2-AID
Up2-Apobec1
Up2-Tet1 CD
Up2-Tet3 CD

Figure 4. DNA methylation level at target promoter upon targeting putative DNA demethylases
In this figure the DNA methylation levels upon targeting putative DNA demethylases is shown. (A) Bisulfite sequencing to determine CpGs of interest for pyrosequencing of ICAM-1 promoter. Each square represents one CpG. Black squares represent methylated CpGs, grey squares represent unmethylated CpGs, white squares are undetermined CpGs. At the top the location of the ZF recognition site (ZF) and the direction of the effector domain (ED) are indicated. (B) Pyrosequencing of CpG #10-14 of the ICAM-1 promoter, showing the average percentage of DNA methylation for each CpG analyzed. Each experiment has been successfully analyzed five or six times. Error bars represent the standard error of the mean. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; paired T-test. (C) Pyrosequencing of CpG #1-4 (see Fig. 1b) of the EpCAM promoter showing the average percentage of methylation. Number of successful analyses per construct are indicated at the top of the graphs. Error bars represent the standard error of the main.

ICAM-1, also CD54-VP64 was able to induce gene expression (average of 88-fold on mRNA, 36-fold on protein level), but CD54-p300 and CD54-UTX did not (Fig. 5b). Also no changes in EpCAM expression were observed in A2780 or Skov3 upon expression of the CD54 ZF only or in fusion to p300 or UTX (data not shown).

In A2780, negative for EpCAM, Up2-VP64 again induced EpCAM expression on mRNA level (average of 3-fold) which could not be observed on protein level due to a low number of surviving cells (Fig. 5c). Up2-p300 did not have any effect on EpCAM expression in A2780. In H134S, negative for EpCAM, Up2-VP64 could not consistently induce EpCAM expression on mRNA level (Fig 5d). When ICAM mRNA expression was assessed, no differences were seen in A2780 and H134S upon expression of the Up2-ZF containing constructs (data not shown).

Gene-specific targeting of histone modifying enzymes: histone modification levels

Although no changes in gene expression were observed for the total cell population, it might still be that histone modification levels are altered due to targeting of p300 or UTX. Upon expression of CD54-p300 in A2780, the intention was to increase H3 and/or H4 acetylation levels on the ICAM-1 promoter, but this was not observed. H3K27me3 levels were slightly decreased at the ICAM-1 promoter in Skov3 cells upon expression of CD54-UTX (Fig. 6a), but this was also the case for the untargeted EpCAM promoter (Fig. 6b). None of the other results were consistent. Upon expression of the Up2 zinc finger constructs, H3 acetylation seems to be increased by Up2-p300 in A2780. However, IgG is also increased (Fig. 6c). No consistent results were obtained by expressing the other constructs, nor for the untargeted ICAM-1 gene (Fig. 6d).

DISCUSSION

In this study, we aimed to induce gene expression of two epigenetically silenced endogenous model genes through Epigenetic Editing. ZF fusions to activation domain VP64 caused induction of gene expression. Targeting of the five tested candidate DNA demethylases did not result in a DNA demethylating effect at the targeted endogenous

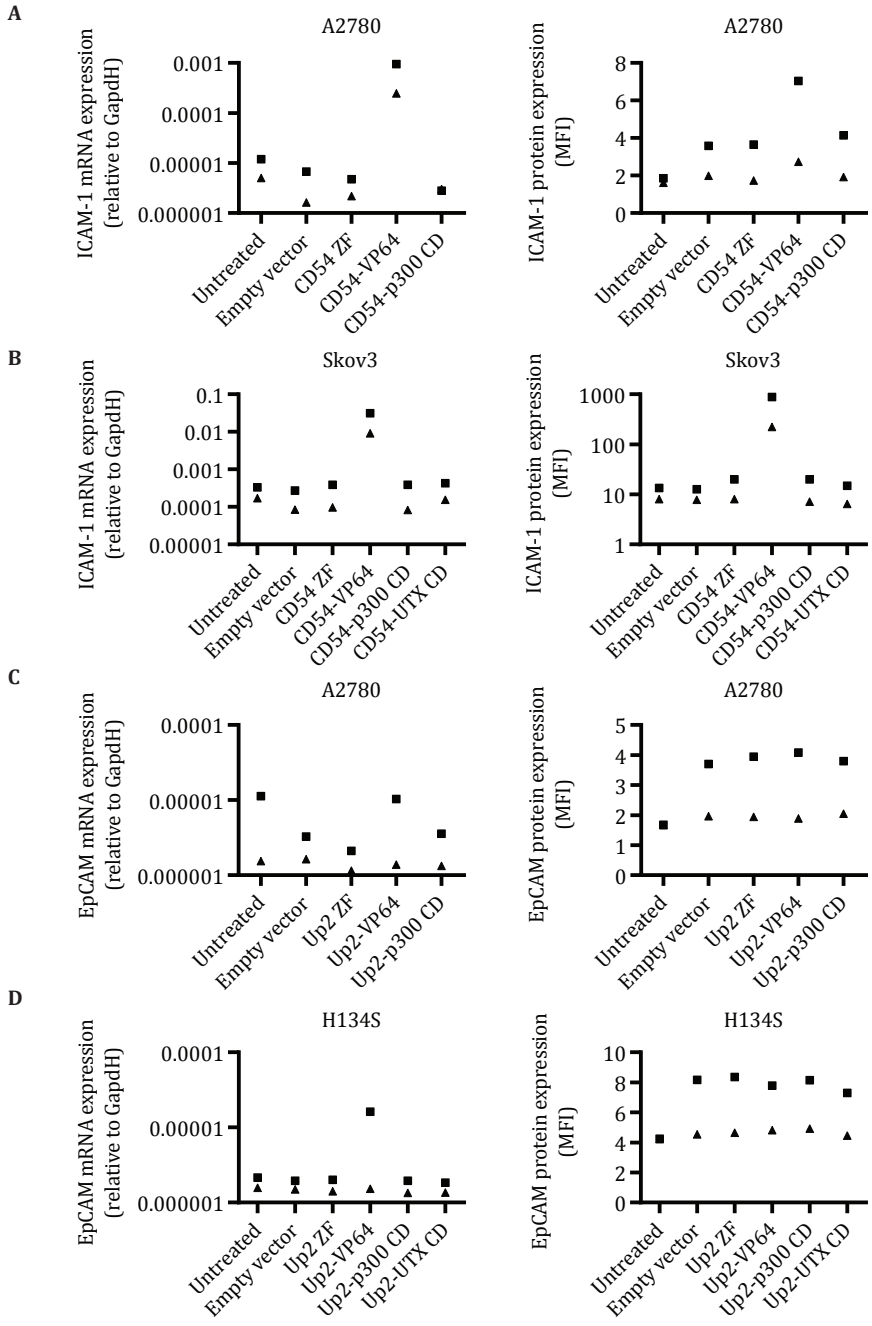

Figure 5. Target gene expression upon targeting catalytic domains of histone modifying enzymes
Expression of ICAM-1 (A, A2780; B, Skov3) or EpCAM (C, A2780; D, H134S) mRNA levels after targeting p300 and/or UTX are represented in the figures on the left side, protein levels in the figures on the right side. Squares represent one experiment, triangles the other experiment (n=2). MFI is mean fluorescence intensity of gated cells.

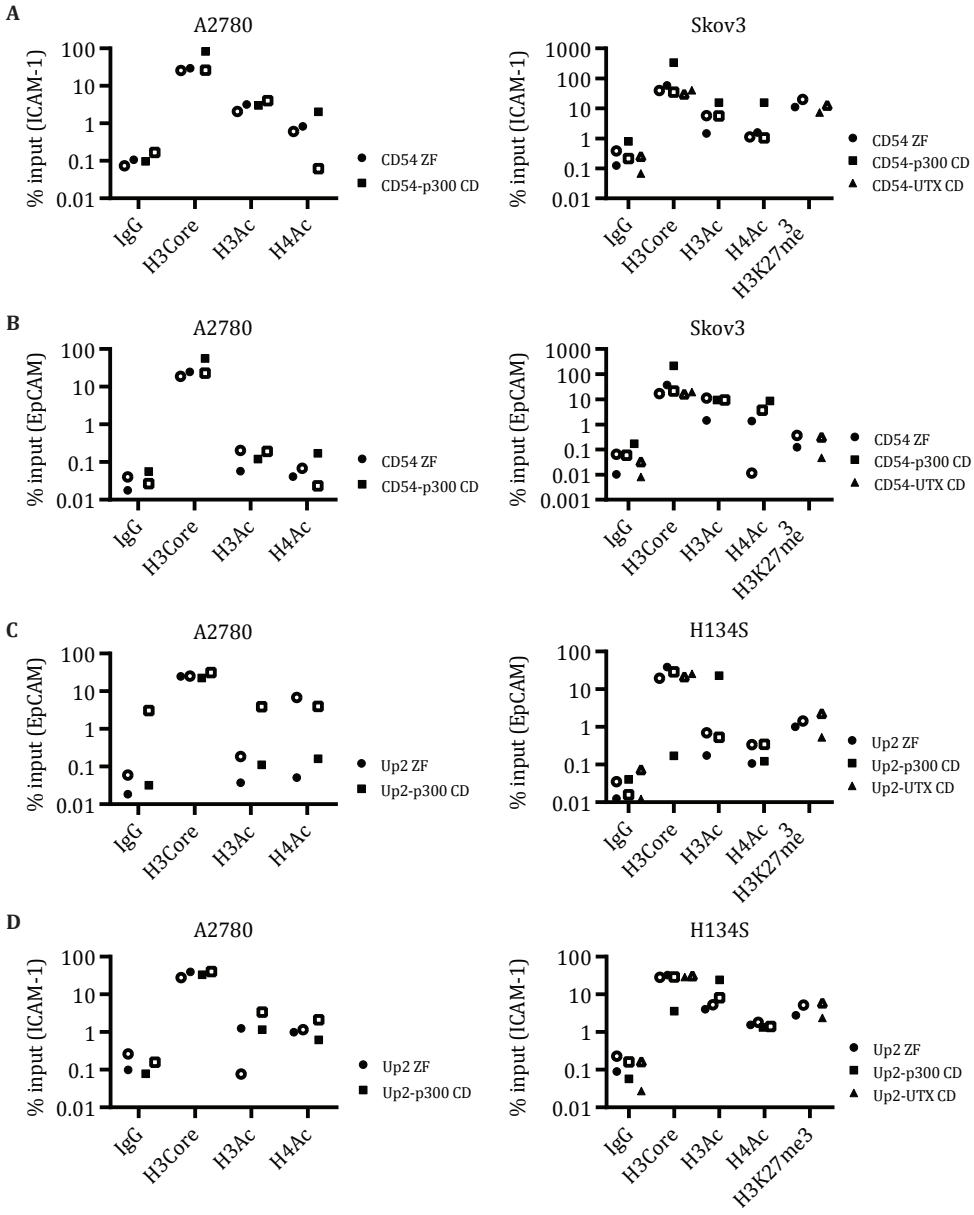

Figure 6. Histone marks with and without targeting catalytic domains of histone modifying enzymes
The percentage of the input of histone modification levels is shown upon targeting catalytic domains of p300 and UTX. Figures show the histone modification levels at the ICAM-1 (A) or EpCAM (B) promoter in A2780 or Skov3 cells upon targeting CD54 fusion proteins and at the EpCAM (C) or ICAM-1 (D) promoter in A2780 or H134S cells upon targeting Up2 fusion proteins. Filled symbols represent one experiment whereas open symbols represent another experiment (n=2).

hypermethylated genes. In agreement, there was a lack of upregulation of expression of either one of the two model genes upon expressing the ZF fusions containing

putative DNA demethylases. In addition, targeting of enzymes known to be capable of altering histone modifications did also not show an effect on molecular level or on gene expression.

As reported by others (40), we observed that targeting of VP64 led to significant hypomethylation of target CpGs in the promoter. Although this might be secondary to the gene expression activation, hypomethylation was also achieved (even to a higher extent) by the ZF only. As both the ZF only and ZF-VP64 construct are highly expressed, an alternative explanation for DNA demethylation by VP64 might be sterical hindrance by the fusion protein, hampering Dnmt1 in copying the methylation pattern to the daughter strand upon cell division. CpGs analyzed in the EpCAM promoter did not show any DNA demethylation upon expression of the Up2 constructs. Although the analyzed CpGs are not situated within the ZF binding site, like for CpG #10 and #11 of ICAM-1, EpCAM CpG #1 and #2 are comparable in distance from the ZF binding site as CpG #12 and #13 of the ICAM-1 promoter. The discrepancy might be explained by context dependency.

Although no additional targeted DNA demethylation was observed by ZF-Tet1 or Tet3 over that observed with ZF only, 5hmC might still have been formed, as bisulfite conversion does not make a distinction between 5mC and 5hmC (41, 42). Therefore, it would be of interest to detect 5hmC levels specifically at the target site, making use of evolving techniques for determining locus-specific 5hmC (43, 44, 45, 46). Previously, when Tet1 was targeted to a site integrated in the genome by fusion to Gal4, this led to downregulation of reporter gene expression (25). However, in that respective study, 5hmC levels were not investigated and the unexpected effect was suggested to be independent of the catalytic activity of Tet1. In fact, it might be (partly) due to the observed recruitment of Sin3a, part of a co-repressor complex. Moreover, the targeted construct was targeted to an active gene which might have caused the lack of detectable (further) gene expression activation. Targeting the enzyme to a hypermethylated reporter gene is more likely to lead to activation of gene expression through the enzymatic activity of Tet1.

No effects of targeted Gadd45α were detected in the current study. Previously, the DNA demethylating effect of Gadd45α observed upon overexpression in one study (47) could not be reproduced by others (48, 49). Moreover, whereas knockdown of the NER machinery, in which Gadd45α is suggested to play a role, resulted in hypermethylation (50), Gadd45α knockout mice do not exhibit the expected hypermethylation (49). However, we have seen induction of reporter gene expression and indications for DNA demethylation by overexpression of Gadd45α or targeting the enzyme to integrated repeats of target sites (chapter 3). Thus, it might be that the targeting of one copy of the enzyme to the target gene is not enough for a significant effect.

Another suggested mechanism of active DNA demethylation includes deamination by AID and Apobec1. Although knock-outs are still viable and fertile (51, 52, 53, 54), which might be explained by redundancy, targeting of AID/Apobec1 is expected to deaminate methylated cytosine, subsequently recruiting mismatch repair enzymes to eventually have the 5-methylcytosine replaced by an unmodified cytosine. However, recently it appeared that deamination of cytosine by AID/Apobec1 is sterically favored over deamination of 5mC and deamination of 5hmC did not even seem to be induced by these enzymes (55). This indicates that the role of AID/Apobec1 in active DNA demethylation might be smaller than previously suggested and would explain the results obtained by us with these domains.

That targeted DNA demethylation is feasible is proven by the recently studied targeting of TDG, a T/G mismatch repair protein (56). This protein, previously suggested to be able to demethylate methylated CpGs (57) or at least to play a role in the DNA demethylation process (21, 58) was targeted to NFκB target sites by fusing it to the NFκB DNA binding domain (56). Indeed, a reduction in methylation levels of 5-10% were observed at the CpGs investigated in that study. Moreover, an effect on gene expression was achieved despite the few CpGs investigated and shown to be demethylated. It might be that DNA demethylation of just one CpG is sufficient for gene expression activation, as for another gene also DNA methylation of just one CpG showed to be sufficient for silencing (59). Such high efficiency is likely due to methylation sensitivity of transcription factors for binding. So, thorough investigation into which CpGs to be targeted could be of importance.

In the TDG study, the targeting construct was delivered via lentiviral transduction and transduced cells were selected by detecting the cotransfected LNGFR protein (56). In some cases, it might thus be necessary to select for transduced cells before analysis. If not all host cells are hit with the virus containing the ZF construct, this might cause an underestimation of the effects. However, this was not reflected in transfection efficiencies determined by the percentage of GFP positive cells (e.g. efficiencies of >90% for the Gadd45α constructs).

Although no changes in gene expression were observed in the present study, this does not necessarily mean that no DNA demethylation has occurred. It is likely that the site of action relative to the TSS is of importance for an effect on gene expression (60). Alternatively, it has been described before that DNA demethylation can be associated with a change in histone modifications, resulting in active histone marks even though this did not lead to a change in gene expression (61).

Besides the lack of targeted DNA demethylation, also for the catalytic domains of histone modifying enzymes no targeted effects were detected in the current study. Amino acids 1066-1707, is the domain of the enzyme that was used for fusion to the

ZFs in this study, since this is also the commercially available HAT domain of p300 and is therefore expected to be able to execute its functions. In other studies, diverse domains of p300 have been targeted to various target sites, as reviewed in (27), resulting in inconsistent outcomes. Some of these studies show targeting of domains that include the one used in this study or are closely similar (62, 63, 64, 65)). From these studies, the domain used in this study seems to be successful in half of the cases. One targeted domain, aa 964-1922 shows activation in one study (62) but no effect in another study (65). In these two studies, the gene expression activating effect that was reported seemed to be dependent on the position of the DNA binding domain target site relative to the TSS, with a downstream binding site being beneficial compared to an upstream target site (62, 65). In this study, both a ZF binding upstream (EpCAM) of the TSS and one binding downstream (ICAM-1) of the TSS of their respective target genes are used. In this regard, the position of the ZF binding site relative to the TSS does not necessarily explain the lack of effect seen in the present study.

UTX, the H3K27 demethylase, has never been reported to be studied in a targeted fashion before, but overexpression was shown to result in a decrease in H3K27 methylation (32). The catalytic activity of the domain used in the present study (aa 401-1401) was effective in the study by Hong et al., as assessed in a cell-free system. Although when targeted to the ICAM-1 or EpCAM promoter no effects were observed, the same domain of UTX did show increase of transcription of a target gene upon single cell analysis in cells containing a large repeat of DBD recognition sites (chapter 3). For UTX, as well as for the other domains, it might be required to target different positions within the same promoter simultaneously.

One general consideration for improvement of the experimental set-up of Epigenetic Editing might be to reduce the size of the retroviral insert to increase expression efficiency. For the ZF fusion proteins in this study (containing UTX CD, p300 or Tet3), the expression is difficult to detect on protein level. Whereas this might be due to technical issues of the read-out, these large proteins are probably expressed to a lesser extent.

Although it has been shown that targeted rewriting of only one epigenetic mark at one locus can be sufficient to cause modulation of gene expression, as reviewed in (27), further improvements to the Epigenetic Editing approach might be made through combination treatment with more than one targeted epigenetic enzyme. This would diminish chances on remaining repressive marks recruiting silencing machinery to regain the repressed state.

Finally, the choice of target gene might influence the likelihood of observing an effect. Obviously, the mark affected by the epigenetic enzyme targeted should be present (or lacking, when inducing a mark). In addition, it could be that ICAM-1 and EpCAM are difficult to target because of their chromatin context. In general, silent chromatin

regions (heterochromatic) are intuitively less accessible for transcription factors than active chromatin regions (euchromatin). This could explain why Epigenetic Editing of endogenous genes so far only has been reported for repression of gene expression (66, 67, 68). However, expression of epigenetically silenced genes, including ICAM-1 (35) and EpCAM (36), was previously induced by targeting VP64 in fusions to ZFs. Moreover, even promoters at the imprinted alleles of genes have been shown to be bound and activated by ZF-VP16 fusions (69). Thus, accessibility does not seem to be an issue. Likely, remaining epigenetic marks recruit repressive enzymes and/or repressive marks are spread. As far as known, no investigation into gene-specific targeting of activating epigenetic enzymes to endogenous epigenetically silenced genes has been reported before.

In conclusion, further investigation is necessary to exclude the proteins targeted in this study as potential candidates. Epigenetic Editing for upregulation of genes needs to be further optimized. When successful, Epigenetic Editing can eventually be of interest for validation of target genes for therapeutic approaches or perhaps even as a therapeutic approach to reactivate epigenetically silenced genes (or to silence overexpressed genes) causing disease.

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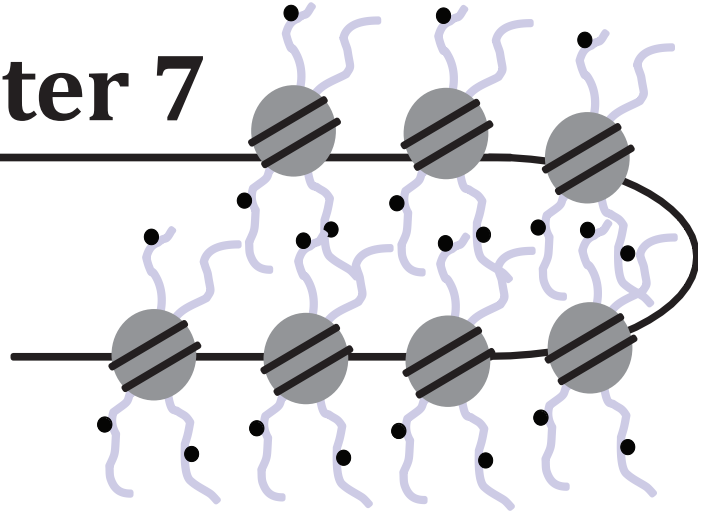
Chapter 7



Summary

AIM

The ultimate aim of the research described in this thesis was to gene-specifically induce gene expression of epigenetically silenced genes through Epigenetic Editing. This can be broken down into two research questions:

1. How can epigenetic marks be rewritten, specifically at the gene of interest?
2. Does this change in epigenetic environment lead to induction of gene expression of the target gene?

CHAPTER 2

After a general introduction in chapter 1, chapter 2 is a review of published research on actively targeted epigenetic enzymes. In most of the studies described in this review, first a piece of synthetic DNA was integrated in the genome of cells. Next, epigenetic enzymes were targeted to this piece of DNA using DNA binding domains recognizing this synthetic DNA. Although much information has been derived from this approach, this synthetic system, unlike zinc finger, is not able to gene-specifically target endogenous genes. Interestingly, in many of the studies described a change in epigenetic marks at the target site or a change in target gene expression was observed. In some cases even both a change in epigenetic marks as gene expression was established. This indicates that the change of an epigenetic mark can indeed directly lead to a change in gene expression, especially since using an enzymatically inactive epigenetic enzyme did not lead to the change in gene expression. Both activation and repression of gene expression was established in the studies described in chapter 2. Even though this seems promising, there are only a few examples of gene-specific targeting of epigenetic enzymes. However, this is of importance to be able to modulate genes of interest and also to prevent the modulation of the epigenetic marks of aspecific genes.

CHAPTER 3

In chapter 3 a study is described in which it was attempted to modulate epigenetic marks and/or gene expression. Towards this aim, an artificial system was used, consisting of cells that include a large amount of target LacO DNA sites. As a DNA binding domain, is this study the LacR protein was used, which can bind LacO DNA. To the LacR protein, proposed epigenetic enzymes were fused, supposedly having an effect on DNA methylation or histone modifications. The epigenetic enzymes used in this thesis all were described to either add activating epigenetic marks or to remove repressive epigenetic marks. Therefore it is thought that the effect of these epigenetic enzymes could lead to activation of gene expression. The usage of a large repeat of target DNA, like in chapter 3, could theoretically lead to an enforced effect, since increased numbers of a certain epigenetic enzyme can be forced on one location simultaneously. In chapter

3, indeed indications were found for some enzymes to be capable of decondensing the DNA. So far this did not lead to significant induction of gene expression when analyzing the whole cell population. However, when the effects were analyzed at single cell level, some indications of induction of target gene expression were found upon targeting Gadd45α (a putative DNA demethylase) and UTX (known as an H3K27 demethylase).

CHAPTER 4

In chapter 3 the effectiveness of certain targeted epigenetic enzymes were examined in an artificial system. However, to accomplish Epigenetic Editing, these epigenetic enzymes eventually need to be targeted to endogenous target genes. This means that the enzymes are targeted to only one target gene instead of to a large repeat of target sites, as in chapter 3.

In chapter 4, the epigenetic environment of a specific gene, EpCAM, was determined in ovarian cancer cells. It appeared that EpCAM is differentially expressed in ovarian cancer cells and that this agrees with the DNA methylation status of the promoter. Also some histone modifications associates with active or passive genes were found to associate with the EpCAM gene. The pattern of these histone modifications, like for DNA methylation, agreed with the expression pattern of the gene. Furthermore, we could show with epigenetic drugs that the EpCAM gene expression could be modulated. Therefore, EpCAM is an interesting model gene for Epigenetic Editing as was attempted in chapter 6.

CHAPTER 5

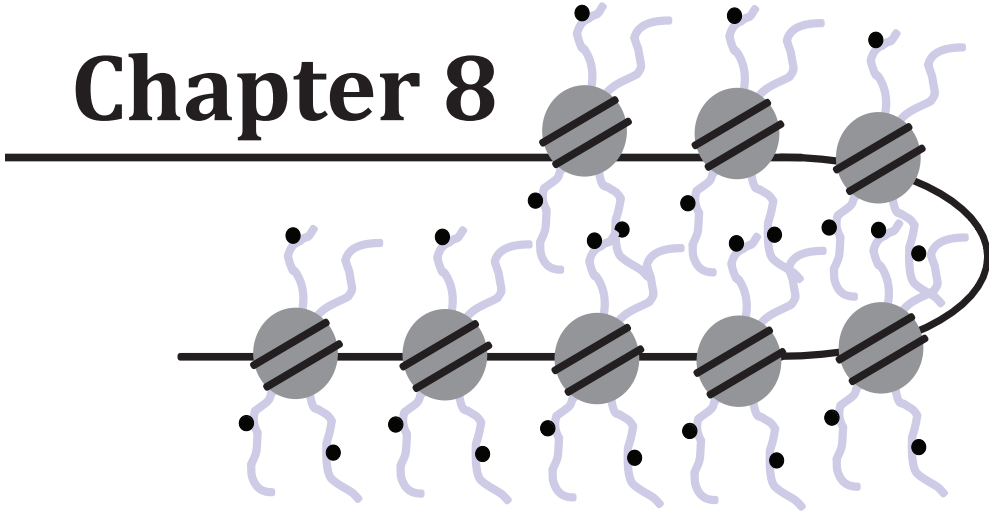
ICAM-1 is another gene that appears to be epigenetically regulated in ovarian cancer cells. In chapter 5 we tested whether published ICAM-1 specific zinc fingers were still able to bind the promoter of this gene when it was epigenetically silenced. Upon fusion of the zinc finger to the activating protein VP64, indeed ICAM-1 expression could be induced. Thus, also the ICAM-1 gene is an interesting model gene for Epigenetic Editing. Not only because it is epigenetically silenced in ovarian cancer cells and we were able to induce gene expression using zinc finger fusion proteins, but also because we observed in chapter 5 that induction of ICAM-1 expression in ovarian cancer cells reduced cell growth.

CHAPTER 6

Just as for ICAM-1, for EpCAM it was previously described that a zinc finger fused to VP64 can induce expression from the epigenetically silenced gene. In chapter 6, we used this information to apply Epigenetic Editing. The epigenetic enzymes described in chapter 3 (and others) were fused to the previously described ICAM-1 and EpCAM zinc

fingers. Upon treating ovarian cancer cells with these fusion proteins, no effect on the targeted epigenetic marks nor on gene expression was observed. This might be caused by several factors, as will be discussed in chapter 8.

Chapter 8



General discussion and future perspectives

INTRODUCTION

In this thesis, the aim was to induce expression of epigenetically silenced genes through gene-specific rewriting of epigenetic marks (Epigenetic Editing). First we screened candidate enzymes for potent epigenetic effector domains, capable of removing epigenetic marks associated with repressed genes or inducing epigenetic marks associated with active genes **(chapter 3)**. We showed that putative DNA demethylase Gadd45α and H3K27 demethylase UTX might represent interesting candidates for reactivation of gene expression through Epigenetic Editing. Subsequently, endogenous model target genes were identified that are epigenetically silenced and could be reactivated with epigenetic drugs **(chapter 4 and 5)**. Moreover, as described by us for EpCAM (1), ICAM-1 expression could be directly induced using Artificial Transcription Factors (ATFs); zinc fingers (ZFs) fused to transient effector domains, in this case transcription activation domain VP64 **(chapter 5)**. Finally, we attempted to reactivate these epigenetically silenced genes using the gene-specific ZFs but in this chapter in fusion to potentially gene expression activating epigenetic enzymes **(chapter 6)**. Although the gene-specific rewriting of epigenetic marks was not achieved, some pitfalls and potentials of Epigenetic Editing become apparent after the performed research and will be discussed here.

PITFALLS

Effective silencing of gene expression has been obtained by targeted DNA methylation (2) or by targeted H3K9 methylation (3) through Epigenetic Editing. The latter was confirmed for another target gene by our lab, using the same delivery system as used in **chapter 6** (4). However, re-expression of an epigenetically silenced gene through Epigenetic Editing (in a gene-specific way) has never been reported. Nonetheless, induction of expression of epigenetically silenced genes has been achieved through other targeting approaches, where sequence-specific DNA binding domains (DBDs) which do not target a specific gene are fused to epigenetic enzymes to enforce their presence on the recognition site(s) of the DBD. **(see chapter 2)**. This indicates that 1) repressed endogenous genes are accessible 2) Overwriting negative marks r inducing positive marks results is associated with an increase in gene expression. Likely, spreading of the activating epigenetic mark is necessary for efficient activation of gene expression. Furthermore, the balance with remaining repressive epigenetic marks, which might recruit endogenous enzymes, needs to be tipped over to an expressive state. In performing this innovative research, we also identified other issues need to be taken into account to improve Epigenetic Editing efforts aiming to achieve induction of gene expression of epigenetically silenced genes, as is described here.

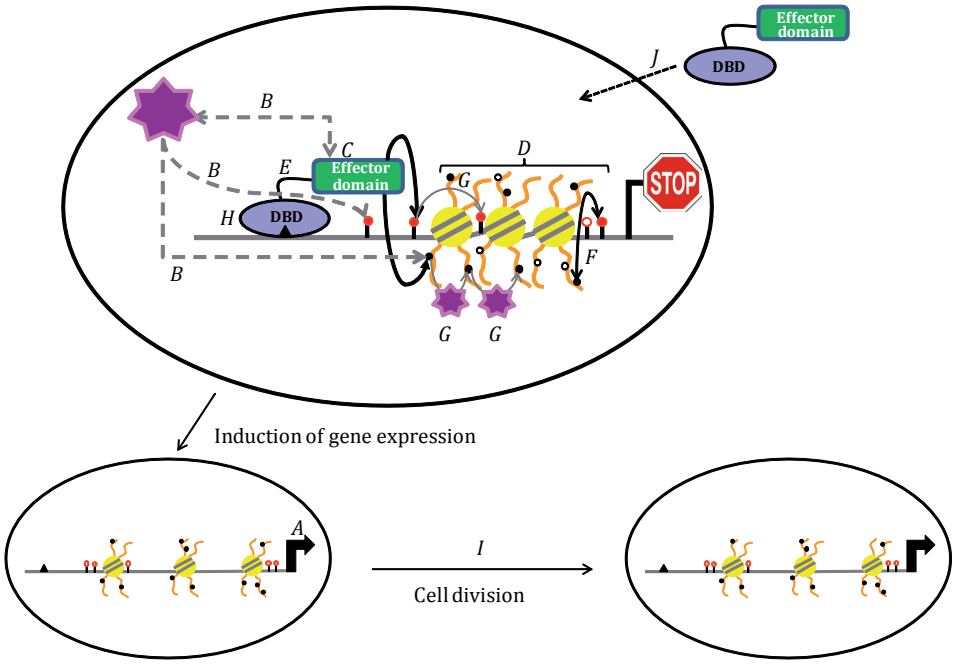


Figure 1. Epigenetic Editing to induce gene expression –pitfalls and potentials

The action of Epigenetic Editing to induce gene expression is schematically shown in this figure. In the upper figure a locus is shown harboring an epigenetically silenced gene. Lollypops represent either unmethylated (open) or methylated (filled red) CpGs. Histones (yellow circles) and their tails (orange) are also represented and histone tail modifications are represented by open or filled black circles (associated with an active or repressed chromatin state, respectively). The DNA binding domain (DBD) recognition site is shown as a black triangle. Indicated are processes that can affect efficiency of Epigenetic Editing: A) Instructiveness of the targeted mark; B) Recruitment of/by other proteins; C) Choice of effector domain; D) Context of the target gene; E)Linker length; F) Crosstalk between epigenetic marks; G) Spreading of epigenetic marks; H) DNA binding domain (targeting device); I) Heritability; J) Delivery.

Instructiveness of the edited marks with respect to gene expression

Genome-wide approaches have resulted in associations of most epigenetic marks with either active or repressed genes. The causality of these marks for the associated gene expression state, however, is in general unclear. Therefore, the feasibility of modulating gene expression by rewriting these marks through Epigenetic Editing is also not clear for most epigenetic marks, target genes and/or target contexts. In particular cases of research into the effect of epigenetic enzymes in targeted approaches **(see chapter 2)** rewriting of one type of mark has been shown to be enough to cause a change in gene expression, as the effect of the enzymes was compared with their catalytically inactive counterpart. Nonetheless, it is not known whether the observed effects on gene expression are actually caused by the epigenetic mark that is changed **(Fig. 1a)**. In fact, in some cases gene expression modulation is achieved by targeted epigenetic

enzymes with an inactive catalytic site (5, 6, 7, 8). It is likely that in these cases the targeted protein recruits other proteins **(Fig. 1b)** that actually perform the effect on gene expression, which are thus of more interest as an epigenetic effector domain for Epigenetic Editing.

In this thesis, we adopted several strategies with the ultimate aim of achieving sustained gene-specific re-expression of epigenetically silenced genes. In addition to targeting candidate DNA demethylases, an H3K27 demethylating enzyme and a histone acetyltransferase have been fused to DNA binding domains (DBDs) to perform their action at predetermined sites **(chapter 3 and chapter 6)**. However, it might be that the specific DNA demethylation, H3K27 demethylation or histone acetylation (alone) is not instructive for re-expression of epigenetically silenced genes.

Theoretically, demethylation of DNA is of interest for activation of gene expression (9). That is, it is known that DNA methylation, in particular at the transcription start site (TSS) and exon 1, is strongly associated with repression of gene expression (10, 11). In this respect, drugs have been developed that inhibit DNA methylation in order to induce gene expression. Such drugs are effective and some are currently used in clinic (12, 13, 14). Whereas until recently mainly hematological malignancies were treated with these drugs, currently also solid tumours seem to respond to DNA methylation inhibitors (15).

However, recently, DNA methylation was reported not to be a stable lock of gene expression, but more of importance for gene silencing memory (16). Furthermore, DNA demethylation does not always seem to be necessary for activation of gene expression, since for some genes (e.g. ICSBP/IRF8 and CAII) promoter methylation (including around the TSS) did not hamper expression when activating histone marks were present (17, 18). Interestingly, even when DNA is demethylated this is not necessarily sufficient for induction of gene expression (19), not even if activating histone modifications are present (which generally seems to coincide) (20).

In addition, there are some indications that DNA demethylation is not instructive for induction of gene expression in all cases, as treatment with e.g. DNA methylation inhibitor 5-aza-2'-deoxycytidine (5-aza) does not lead to re-expression of all methylated genes. In this respect, it was shown that upregulation of genes upon treatment with 5-aza depends on the CpG content and methylation rates at the specific promoters (21). Moreover, treatment with DNA methylation inhibiting drugs was shown to cause as many genes to be upregulated as downregulated (22). Furthermore, DNA demethylation, through treatment with 5-aza or deletion of the DNA methyltransferases, does not seem to be sufficient for complete transcriptional (re-)activation, which is suggested to be caused for example by a lack of recruitment of Dot1L, responsible for H3K79me2 (23).

However, in another example, demethylation of DNA by inhibiting the DNA

methyltransferases resulted in activation of gene expression, followed by a change in histone modifications to a more permissive chromatin state (24). Importantly, endogenous targeting of multiple genes by a candidate DNA demethylase Thymine DNA Glycosylase (TDG) upon fusion to the NFκB DBD did result in DNA demethylation and, moreover, induction of target gene expression (25). This indicates that, at least in particular cases, DNA demethylation can be instructive for reactivation of expression of epigenetically silenced genes.

Less is known about the instructiveness of H3K27 demethylation with respect to gene expression activation. It is reported that UTX, the H3K27 demethylase used in this thesis **(chapter 3 and 6)**, is needed for induction of pluripotency in induced pluripotent stem cells, but not so much for maintenance of the feature (26). This could indicate that H3K27 demethylation is less suitable for long-term re-expression of epigenetically silenced genes by Epigenetic Editing. Another study shows that H3K27 demethylation is essential for normal development through the activation of gene expression (27). Also, mutations in UTX are associated with cancer and reintroduction of expression of UTX not only resulted in reduced proliferation, but also activation of gene expression (28).

Histone acetylation is often associated with active genes. Nonetheless, there are indications that histone acetylation is not predictive and thus might not be instructive for activation of gene expression (29). Interestingly, in targeted studies, histone acetyltransferases seem to effectively induce gene expression. p300, one of the previously targeted histone acetyltransferases is, as far as known, the only enzyme of the ones used in this thesis **(chapter 6)** that resulted in targeted induction of reporter gene expression before (see Table 2b of **chapter 2** (30)). Thus far, only one study exploiting targeted histone acetyltransferases investigated and showed both induced histone acetylation and gene expression, in the particular case by targeting p300 (31). Although this study investigated the effects on plasmid level with cotransfections, it might be that the effect can be translated to the instructiveness of targeted histone acetylation in the endogenous chromatin context. The other targeting studies all show activation of gene expression. Interestingly, two of these targeting studies enforce presence of histone acetyltransferases on epigenetically silenced endogenous targets by fusing the histone acetyltransferases to the methyl binding domain (MBD) of MeCP2 or to the DNA binding domain of MLL. This might indicate that histone acetyltransferases are good candidates to use in Epigenetic Editing, although we were not able to show this thus far. Nonetheless, more investigation is needed to ensure that the observed targeted effects on gene expression are indeed caused by induction of histone acetylation. Also issues like heritability of the effect have not been addressed leaving the applicability of these enzymes for long-term induction of gene expression uncertain.

Activity of (domains of) epigenetic enzymes

In determining the choice of the epigenetic effector domain **(Fig. 1c)** for Epigenetic Editing, there are other challenges in addition to predicting the instructiveness of its actions. In **chapter 3** and **chapter 6** we attempted to identify true DNA demethylases by overexpression and by fusing putative DNA demethylating enzymes or domains thereof to DBDs. As far as size limitations allow, it is possible to target full length epigenetic enzymes, which is (partially) dependent on the delivery method. However, in some sequence-specific targeting studies reviewed in **chapter 2**, it was shown that certain domains of epigenetic enzymes are more active than full length enzymes. Moreover, in some cases the full length protein was not active at all. As an example, full length Suv39H1 did not result in gene repression upon fusion to a ZF (3). In this particular case, the lack of effect was suggested to be caused by capturing of the fusion proteins by endogenous proteins like HP1. Evidently, this problem might occur for many DBD-fused and also untargeted enzymes. Thus, it seems worthwhile to try out several truncations of the enzyme of interest. For true Epigenetic Editing, rewriting the epigenetic mark at the target gene, targeting of the catalytic domain of the enzymes should be sufficient. In this thesis, in **chapter 3 and/or 6,** the full length of Gadd45α, Aid (murine) and Apobec (murine) were fused to DBDs. As such, they might possess domains actively recruited by endogenous proteins (like suggested for Suv39H1), hampering the fusion proteins from reaching their target site. This, however, does not seem to be the case for Gadd45α as it clearly showed decondensation and an increased transcription upon targeting to a LacO repeat integrated in cells upon single cell analysis **(chapter 3)**.

With Epigenetic Editing, theoretically only two copies of DNA need to be targeted and thus only two copies of the Epigenetic Editor are expected to be needed per cell. However, expression efficiency might be of influence on the activity and this could also be caused by the (size of) the effector domain fused, as was seen in both **chapter 3 and 6**. The larger effector domains (UTX, p300, Tet1 and Tet3) showed little or no expression. However, UTX was effective in fusion to LacR, despite the lack of detectable protein expression.

Tet proteins

For Tet1 and Tet3 (both murine), truncated domains were used, which are the CXXC domains, found to be active for human Tet1 by Tahiliani et al. (32) and for murine by He et al. (33). These catalytic domains were previously reported to be active in oxidation of 5-methylcytosine (5mC) upon overexpression. In addition, Tet1 was previously studied in a targeted fashion, although the exact amino acids used in the respective Gal4 targeting study are unclear (34). The targeting of Tet1 in the study by Williams et al., interestingly resulted in repression of the targeted reporter gene,

but DNA (hydroxy)methylation levels were not investigated and Tet1 was targeted to an active reporter gene. Importantly, the Tet proteins facilitate conversion from methylated to hydroxymethylated cytosine, and further oxidation to 5-formylctosine and 5-carboxylcytosine. Further conversion to unmethylated cytosine is demonstrated to be performed by TDG (33, 35). Nevertheless, as hydroxymethylated cytosine levels were not determined in this thesis, no conclusions can be drawn about the oxidation activity of the targeted Tet domains in **chapter 6**. Whereas bisulfite treatment does not result in a distinction between 5mC and 5-hydroxymethylcytosine (5hmC), currently several assays are reported to investigate hydroxymethylation levels in a locus-specific manner. The most interesting assay if the exact target CpG(s) are unknown is an adjusted bisulfite sequencing protocol enabling analysis at the single base level (36, 37). However, as 5-hmC antibodies are now available, hMeDIP, the detection of immunoprecipitated DNA associated with 5-hmC, might be sensitive enough when the target CpG is known.

Ros1 and Demeter

In contrast to the Tet proteins (of which the catalytic domains used were previously reported to be active), for Ros1 (aa 868-1105) and Demeter (DME; aa 1189-1418), investigated in **chapter 3,** truncations of the genes were made based on homology to each other and Demeter-like proteins DML-2 and DML-3 (38), which could explain the lack of their activity. Since the truncated Ros1 and DME were only investigated upon untargeted overexpression, it might be that some essential domains are missing, like their DBD, causing the lack of effect. These truncations of Ros1 and DME might however still be effective upon fusion to DBDs. Despite the fact that the activity of these plant DNA demethylases have not been examined in mammalian cells before (to the best of our knowledge), plant Suv39H1 has been successfully targeted to a mammalian gene (3). Also the prokaryotic DNA methyltransferases M.SssI and M.HpaII were successful in targeted methylation of yeast and mammalian DNA, respectively (39, 40, 41). This indicates that certain epigenetic mechanisms are conserved throughout eukaryotes and, moreover, the feasibility of using enzymes from other species for Epigenetic Editing in mammalian cells. It could be necessary to optimize the codon usage to achieve efficient expression in mammalian cells.

AID/Apobec1

For the candidate DNA demethylating enzymes used in this thesis, only circumstantial evidence indicates that they are indeed able to cause DNA demethylation (42). Increasing amounts of evidence indicate that it is likely that the mammalian DNA demethylation mechanism is a cascade of events, involving multiple proteins. Several suggestions hint towards repair related mechanisms (base excision repair/nucleotide excision repair)

(43, 44, 45, 46, 47). Even though direct 'repair' of 5meC/G to C/G might be possible, it seems more likely that the 5meC is first converted to another intermediate, such as a thymine through deamination or to 5hmC through hydroxylation. Interestingly, Aid and Apobec1 (deaminases previously suggested to be part of the DNA demethylation process (48, 49, 50)) have recently been shown to be more likely deaminating cytosines than 5mC, while deamination of 5hmC was undetectable (51). This indicates that if deamination plays a role in active DNA demethylation, it is likely that the pathway in which they are involved is independent of the pathway involving oxidation of 5mC to 5hmC. Targeting of these deaminases to endogenous genes did not result in DNA demethylation or gene-activation **(chapter 6)**, but further investigation is necessary to detect whether deamination did take place.

Gadd45α

The most promising potentially DNA demethylating enzyme in this thesis is Gadd45α. We were able to show induction of reporter gene expression in cotransfections where Gadd45α was overexpressed **(chapter 3)**. Moreover, LacR-fused Gadd45α appeared to be able to induce chromatin decondensation and transcription of a reporter gene integrated in mammalian cells **(chapter 3)**. However, no effect of the enzyme was observed when targeted gene-specifically to endogenous genes by fusion to ZFs **(chapter 6)**. This might be explained by the fact that the targeted effect seen upon fusion of Gadd45α to LacR was achieved when targeting to a repeat of LacO sites and analyzing at single cell level. Thus, it might be necessary to design more than one ZF, all binding close to each other in one same region, to obtain significant effects. Another option is to look at single cells for DNA demethylating effects. In addition, there has been some debate on whether Gadd45α indeed plays a role in active DNA demethylation or not. Whereas in first instance Gadd45α was thought to induce the first step of DNA demethylation (52), later the protein was suggested to be more of importance for the formation of protein complexes, likely consisting of deaminases and DNA repair proteins (43, 49). The exact mechanism of DNA demethylation involving Gadd45α needs further investigation, which might be achieved through assays like ChIP-reChIP. In this way, the proteins recruited by Gadd45α can be determined, potentially leading to new interesting candidates for fusing to DBDs in Epigenetic Editing attempts.

UTX

The domain of the H3K27 demethylase UTX (aa 401-1401) that was used in targeted efforts in **chapter 3 and 6** has not been targeted before. In fact, the activity of this truncated domain was only reported upon overexpression after which the nuclear cell extracts were incubated with calf core histones (53). Nonetheless, like for Gadd45α,

also for UTX effects were observed when analyzing single cells and upon targeting to a large repeat of DBD recognition sites **(chapter 3)**. Again, no significant effects on levels of molecular epigenetic marks or on gene expression were observed upon fusion of the domain to ZFs and targeting to endogenous epigenetically silenced genes **(chapter 6)**. It might be that there is more H3K27me3 present in the cells comprising the LacO repeats that were analyzed on single cell level, but this has not been assessed. Again, also transfection efficiency and the fact that only one copy of the enzyme can be targeted to the ZF binding site might explain the lack of effects seen in **chapter 6**.

p300

The HAT domain of histone acetyltransferase p300, chosen for targeted induction of histone acetylation in this thesis **(chapter 6)**, consists of the same amino acids as used to produce a commercially available variant of the catalytic domain of the enzyme. As described above, several domains of the histone acetyltransferase p300 (of which some including the domain used in this thesis or are closely similar (54, 55, 56, 57)) have previously been targeted to predetermined sites, mainly by fusion to Gal4 (see **chapter 2, Fig. 2**). From these studies, the domain used in this thesis seems to be successful in half of the cases. One targeted domain, aa 964-1922 shows activation in one study (54) but no effect in another study (57). Here it appears that the position relative to the TSS is of importance. The study where activation was observed was where the target site was downstream of the TSS. Although this is also the case for the ZF binding site in the ICAM-1 promoter **(chapter 5 and 6)**, no effect was observed upon expressing the ZF fused p300. Importantly, the study showing the activation by targeting p300 to a site downstream of the TSS was performed with cotransfections. It might be that the position dependency is different in the endogenous chromatin context. Furthermore, the chromatin environments targeted in **chapter 6** already are occupied by some histone acetylation **(see chapter 4 and 5)**, despite the repressed state of gene expression. As targeting of p300 could not show a further increase of acetylation levels, no effect on gene expression was observed, nor expected. This domain was not investigated upon fusion to LacR, which might be of interest as other domains unsuccessful in Epigenetic Editing (Gadd45α, UTX CD) to endogenous genes were successful upon single cell analysis when targeting to LacO repeats **(chapter 3)**.

The context of the Epigenetic Editing target gene

In chapter 4 and chapter 5 we validated two model genes for induction of gene expression through Epigenetic Editing in chapter 6. These genes (Epithelial Cell Adhesion Molecule; EpCAM and Intercellular Adhesion Molecule-1; ICAM-1, respectively) showed to be epigenetically silenced in certain ovarian cancer cell types in addition to the fact

that ZFs targeting these genes were already published. For effective Epigenetic Editing, certain factors with regard to the target gene are likely to be of importance and could be a pitfall. For example, the chromatin environment of the target gene needs to be able to accommodate Epigenetic Editing (Fig. 1d).

Targeting a DNA demethylase to a gene that is not methylated obviously is unlikely to cause increase of target gene expression, as could be seen upon targeting of Tet1 to an active reporter gene (34). However, the degree of, for example, DNA methylation that needs to be demethylated by a DNA demethylase before it exerts an effect on gene expression is unknown. Moreover, it might be that the presence of other marks than the one addressed hampers the execution of the desired effect. For instance, H3K4 methylation marks prevent DNA from being methylated (58, 59, 60). Although DNA methylation and H3K4 methylation have been detected at the same gene, they were not present at the exact same site (61). Furthermore, some marks are mutually exclusive, such as methylation and acetylation of lysine residues (62), or methylation of H3K4 and H3K9 methylation (63). So, for long-term activation of gene expression, it could be worthwhile to induce H3K4 methylation in order to prevent repression through DNA methylation or to induce histone acetylation to prevent the repressive histone mark H3K9 methylation from being induced.

Also DNA methylation and H3K27 methylation have been shown to be mutually exclusive at an imprinted gene (64). In another study, the silencing through H3K27 methylation seems to be independent of DNA methylation (65). Contrastingly, another report shows dependence of DNA methylation on H3K27 methylation (66). This variability of results can be explained by cell type or gene-specific factors. In the study showing that DNA methylation and H3K27 methylation are mutually exclusive at an imprinted gene, H3K9 methylation appeared to be a prerequisite for proper induction of DNA methylation (64), which has been described more often (67). Although H3K27, H3K9 and DNA methylation are all associated with repressed genes, this sort of information might also be of importance for achieving induction of gene expression through Epigenetic Editing. In those cases where it is known that marks are mutually exclusive, it is likely essential to first remove the hampering epigenetic mark before the mark of interest can be influenced.

Noteworthy, it might be that ChIP assays give results on histone tail modifications more upstream or downstream of the CpGs that were analyzed for methylation. In this respect, BisChIP-seq has recently been introduced; a way to simultaneously analyze histone modifications and DNA methylation at a location of interest (68, 69). This technique could also come in handy to analyze the epigenetic context of a certain gene before and after intervening via Epigenetic Editing.

Another issue is the distance of the recognition sequence of the DBD in relation to

the mark desired to modulate as was investigated for ZF-VP16 fusion proteins when targeted to reporter genes integrated in the genome of plant cells (70). In this study it was shown that a ZF-VP16 fusion protein is most effective when targeted to a site as close to the TSS as possible. Targeting to a site downstream of the TSS, however, leads to blocking of transcription, probably by hampering the activity of RNA polymerase. However, in mammalian cells this does not need to be the case, as ZF-VP64 fusion proteins were able to induce ICAM-1 expression upon binding to a site downstream of the TSS **(chapter 5)**. ZF-fusions to DNA methyltransferases also showed certain preferences in the distance between DBD and target of activity (71, 72). Although the linker length **(Fig. 1e)** between ZF and effector domain can be varied, which might facilitate flexibility of the targeting construct, increasing flexibility can also lead to an unintended effect on physically close parts of DNA. In general, more systemic research into such elementary factors as DBD-target distance should be executed.

It is tempting to assume that due to the compact chromatin formation, DBDs can not reach epigenetically silenced genes. This would indicate that induction of gene expression of epigenetically silenced genes is more challenging than repressing gene expression through Epigenetic Editing. Indeed, Epigenetic Editing in order to induce gene expression has not been shown before. However, upon fusion of gene-specific DBDs to transient transcriptional activators such as VP16/64 or p65, induction of gene-expression can be observed. Even when imprinted genes are targeted, ZFs are able to reach their recognition site within this heterochromatic regions (73). In addition, even large macromolecules are able to access condensed chromatin regions (74).

Cross-talk reinforces silencing but prevents activation

As becomes clear from the above, epigenetic marks communicate **(Fig. 1f)**, either reinforcing a state of chromatin or preventing a state of chromatin from being induced. In this respect, changing one epigenetic mark might be sufficient for it to cause a cascade of effects eventually resulting in enforced change of gene expression in the desired direction. However, upon changing just one mark, this cross-talk of epigenetic marks, their writers and readers, might on the other hand cause reinstallment of the original epigenetic status after the epigenetic editor is cleared from the system.

Such cross talk can have an effect on the order of events needed to induce or repress gene expression. In Arabidopsis, where the mechanism of DNA demethylation is known, it was shown that a certain protein (IDM1) binds DNA in a repressive chromatin state, containing DNA methylation and lacking H3K4 methylation. By subsequently facilitating H3 acetylation, the chromatin state becomes permissive for DNA demethylation to take place (75). A similar sequence of events might also occur in the mammalian situation. In this respect, it might be necessary to first remove H3K4 methylation or add H3

acetylation before DNA methylation marks can be modified. Thus, subsequent targeting of e.g. p300 and a DNA demethylase might be successful.

Some reports already give indications on the possible order of events in mammalian cells. For example, upon investigation of the silencing of stably integrated transgenes, it was observed that H3 and H4 are first hypoacetylated, together with a reduction of H3K4me3 (76). The later observed H3K9 methylation and DNA methylation apparently are not the first steps in such frequent events of gene silencing. To prevent transgene silencing, targeting histone deacetylases and/or H3K4 demethylases would likely be effective. Indeed, targeting of p300 specifically to reporter genes integrated in mammalian cells was previously shown to prevent silencing (55).

Crosstalk might occur at several levels. It can be that histone modifications communicate within a histone tail, within one nucleosome, between two adjacent nucleosomes or between two more distant nucleosomes (77). Various examples of crosstalk between epigenetic modifications have been described, as extensively reviewed in (78). An advantage of crosstalk is that it is also important for the general phenomenon of spreading **(Fig. 1g)** of an epigenetic mark. A well known example of this is recruitment of HP1 by H3K9 methylation, subsequently recruiting H3K9 methyltransferases that spread the mark and can recruit DNA methyltransferases ro reinforce the repressed state. An example of crosstalk to spread epigenetic marks is for example discussed for the ß-globin locus in (79).

POTENTIALS

Although there are some pitfalls that need to be avoided (as described above), there are also many potentials for Epigenetic Editing. Recently, successful efforts have been reported (2, 4) and future research can increase efficiency, eventually resulting in a wide range of applications for the approach.

Gene specificity

Upon finding a good way to perform Epigenetic Editing, no matter what the application is, it is detrimental to validate the assumed gene-specificity of the approach. In theory, the number of ZFs stitched together has a big influence on the specificity and six ZFs, targeting 18 base pairs (bp) is a mathematically unique address in the human genome (80). However, increasing the number of ZFs might, in addition to possibly increase the specificity, also decrease the efficiency. For some genes it could be that five (or less) ZFs are enough to target only that specific gene, which will also be dependent on other factors such as accessibility of the DNA at the target site compared to potential off-target recognition sites. In this regard, clinical trials have been executed with ZF proteins comprising three fingers, without leading to toxicity (81).

Recently, ChIP-seq, the genome-wide sequencing of immunoprecipitated chromatin, is a technique that is more and more used. As far as known, no ChIP-seq studies were reported to prove gene-specific DNA binding of engineered ZFs. One report did show ChIP-seq of a naturally occurring ZF with nine fingers fused to a repression domain, which appeared to bind over 5000 sites (82). Apparently, not all nine fingers bound in this case. It could be that the off-target effects are caused by binding of different combinations of only part of the nine fingers. Moreover, it can be that the ZF fusion protein was recruited to other sites because of the effector domain (KRAB) fused. Although additional ChIP-seq studies on the gene-specificity of ZF binding would be interesting, one might argue that relatively little background binding will be lost with this assay, as very small peaks will be filtered out with regard to high peaks of gene-specific binding. In addition, whereas gene-specific binding is nice and could be essential, it is more important to establish a gene-specific effect on gene expression, which is the desired ultimate outcome. Indeed, upon targeting a ZF-TF with a repressive effector domain, DNA microarrays were performed showing that only the target gene was silenced (83). However, with regard to specificity of ZF binding, mainly prediction methods have been reported (84, 85).

In **chapter 2** we also touched upon the possibility of creating less active mutants of the epigenetic enzymes or using the split enzyme approach (86). Both approaches are designed to ensure that the epigenetic enzyme does not exert its effect before reaching its intended target site through binding of the DBD.

Targeting device: Epigenetic Editing is flexible in using DNA binding domains

In this thesis, epigenetic effector domains were targeted to predetermined site either in a sequence-specific way, through fusion to LacR **(chapter 3)**, or in a gene-specific way, through binding to ZFs **(chapter 3 and 6)**. Whereas significant effects were obtained with the LacR DBD, this can not be exploited for use in approaches where gene-specificity is needed.

In addition to ZFs (used in **chapter 3, 5, 6**), other engineerable gene-specific DBDs have been reported, which might be of interest for Epigenetic Editing **(Fig. 1h)**. Polyamides and Triple helix forming oligonucleotides (TFOs) were also used to modulate gene expression (87) Disadvantages of these domains in comparison to ZFs are that TFOs only efficiently bind purine-rich sequences, limiting the choice of target sites. TFOs and polyamides thus far primarily showed effects on transcription by blocking transcription factor binding sites, causing repression or activation. However, a TFO targeting the EpCAM promoter proved to be able to target DNA methylation to a predetermined site of a reporter plasmid upon fusion to a methyltransferase (88). Also, the short stretches of polyamides might be useful because of their relatively easy

nuclear uptake (87).

Recently, increasing attention is focusing on new DBDs; the Transcription Activation-Like (TAL) effectors. These domains are derived from plants and have already shown their effect on target gene expression upon fusion to VP16 or VP64 (see (89) and references therein). As opposed to ZFs, the effect of TALes seems to be context independent. Where a ZF protein consisting of multiple fingers, each potentially influencing specificity, this does not seem to be the case for TALes. Like for ZFs, the influence of the epigenetic environment at the target site on TALe binding is currently still unknown (90). An advantage of TALes is that they appear to be relatively cheap, easy and quick to make versus commercially engineered ZFs (91). In comparison to ZFs, TALes recognize only one bp whereas ZFs recognize three bp. So in order to target a unique address in the human genome, you need to stitch together more TALes, but this likely makes the approach more flexible. However, for a TALe to recognize one bp, it needs 34 amino acids (92, 93), whereas a ZF consisting of approximately 30 amino acids recognizes three bp. As far as current research can show, TALes seem more specific (and thus less toxic). This might be due to the fact that one finger in a multimer of ZFs can influence specificity whereas TALe monomers do not seem to have this disadvantageous property. However, an aspect that might be a major disadvantage of the use of TALes is the delivery into (the nucleus of) the cell because of its size (94).

Heritability

One of the less recent definitions of epigenetic is: "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence", as quoted in (95). Thus, one of the great potentials of Epigenetic Editing is long-term modulation of gene expression, because epigenetic marks are mitotically stable (inherited to daughter cells). Depending on the application of Epigenetic Editing, this heritability is of importance **(Fig. 1i)**. Whereas ATFs, combination of ZFs with transcription activating or repressing domains such as VP16 and KRAB, respectively, have been extensively used to successfully modulate gene expression, this is a transient solution as the domains do not have enzymatic activity themselves. However, when swapping the effector domains for epigenetic enzymes, this could lead to sustained gene expression modulation.

Although for DNA methylation the mechanism of inheritance is relatively well established (96), for histone modifications the mechanisms are currently not completely clear. Nonetheless, evidence for mitotic inheritance of histone modifications is there (97, 98, 99, 100, 101) and various models for the mechanism of mitotic inheritance have been put forward (97, 98).

Delivery

With respect to delivery, ZF fusion proteins have been delivered to cells in different ways. Whereas plasmid transfection or retroviral transduction has been used in most cases (as also in **chapter 3, 5 and 6**), other systems are possible **(Fig. 1j)**. Recently, ZF fusion proteins were also delivered in RNA (102) and protein forms (103). In addition to the form in which the Epigenetic Editors will be forced into the cells, also methods should be investigated as to how the Epigenetic Editors can only reach the tissue or cell-type of interest. If taking cancer as an example, induction of tumor suppressor genes in off-target cells or tissues does not necessarily need to be a problem. However, a gene that is a tumor suppressor gene in one cell-type might be an oncogene in another type of cells. This is nicely exemplified by the EpCAM gene, expression of which has different effects depending on tumor type (104). Ways to ensure cell-type specific delivery of Epigenetic Editors include the use of complexes of antibodies recognizing specific cells or tissue and a cationic lipid or liposomes (105). However, such approaches need further research.

Applications for Epigenetic Editing

Epigenetic Editing can cause modulation of endogenous gene expression when the optimal circumstances are identified. Therefore, in first instance, Epigenetic Editing is useful as a tool to investigate the function of a gene in great detail and with a controllable system. That is, in contrast to overexpression of genes, Epigenetic Editing ensures re-expression of all isoforms of a gene, in natural ratios. When the aim is to repress target genes, Epigenetic Editing has as advantage over e.g. siRNA that it only needs to target two copies of the gene at DNA level, as opposed to targeting a large amount of constantly produced RNA or difficult to reach protein molecules. Subsequently, target genes can be validated for their potentials as therapeutic targets. In this respect, we showed that repression of ICAM-1 expression might play a role in the ovarian cancer tumorigenicity, as gene-specific induction resulted in tumor growth inhibition **(chapter 5)**. Importantly, Epigenetic Editing is not cell or tissue-type restrained and can thus be deployed in every model system of interest.

Furthermore, Epigenetic Editing is a powerful tool to answer fundamental questions on the consequence of epigenetic marks on gene expression. Especially upon comparing effects of certain targeted epigenetic enzymes with their catalytically dead mutants, such answers can be obtained. Also features like the inheritability and spreading of epigenetic marks could be investigated in more detail.

Eventually, Epigenetic editing might be of interest as a therapeutic approach. When re-expression of epigenetically silenced genes is achieved, treatment of a variety of diseases becomes within reach. Although by now there are many diseases (including

neurological diseases like Alzheimer and Parkinson (106), cardiovascular disease (107) and allergic diseases like asthma (108)) known in which aberrant epigenetic silencing plays an important (causing) role, most research has been performed in cancer. Here, tumor suppressor genes more frequently appear to be aberrantly epigenetically silenced than genetically mutated (109). Interestingly, it was shown that silencing through DNA methylation of only two tumor suppressor genes is sufficient to cause cancer formation (110). Interestingly, this might indicate that also the reversal of silencing of few tumor suppressor genes might alleviate cancer. Indeed, through siRNA, cDNA or ATF treatments it has been shown for numerous genes that silencing or activation of one gene already led to a significant reduction in tumorigenicity ((111), including for ICAM-1 in **chapter 5**).

FUTURE PERSPECTIVES

In conclusion, whereas the general aim of this thesis – Epigenetic Editing to induce expression of epigenetically silenced genes - was not fully achieved, possibilities for further research into optimizing the Epigenetic Editing protocol are identified.

It is likely that more than one mammalian active DNA demethylation mechanism exists. Moreover, probably more than one targeted protein is needed before activation of gene expression is achieved. Thus, it might be necessary to cause co-presence of multiple proteins at the target site. In some cases targeting of the enzyme causing the first step in the cascade (like the Tet proteins, hydroxylating 5mC), subsequently recruiting endogenous cofactors (such as TDG) to complete the process might be sufficient. However, it might also be that more than one protein needs to be targeted to the same target site for the proteins to find each other, in collaboration leading to activation of gene expression. This could be achieved by *1)* expressing the DBD in fusion to two (or more) different epigenetic effector domains, acting after each other *2)* expressing one DBD with two different enzymes fused (e.g. N- and C-terminally) *3)* expressing two different DBDs, both binding in the same target region, fused to different epigenetic effector domains. In general, the targeting of more than one epigenetic enzyme might be beneficial, for example the combination of a DNA methyltransferase and a histone modifying enzymes. In this regard, also combination therapies of a DNA methyltransferase inhibitor and a histone deacetylase inhibitor showed promising results (15).

Importantly, the context of the target gene needs to be taken into account, as the effect of several epigenetic effectors is shown to be context dependent. The efficiency of targeting a particular epigenetic effector domain with respect to induction of gene expression might be esteemed by treating cells with epigenetic drugs. Upon induction of gene expression in that way, epigenetic marks could be analyzed to observe which

effects lead to induction of gene expression.

Furthermore, the detection of effects could be improved for example by sorting or selecting transfected cells (as was also performed by e.g. (25)), to make sure all cells that are analyzed indeed express the construct, as we show that when analyzing at single cell level, effects are more detectable. Another option would be to immunoprecipitate DNA associated with the construct (by ChIP of the HA-tagged zinc finger proteins) followed by a method to investigate the changes in the epigenetic mark of interest, to ensure presence of the epigenetic editor at the target site.

With an epigenetic effector domain that is able to rewrite the epigenetic mark and subsequently lead to gene expression modulation, systematic experiments should be executed to optimize the approach. In such experiments optimal linker length and/or distance between DBD and epigenetic effector domain can be assessed. Also issues like heritability and spreading of the epigenetic marks need to be further investigated.

Other domains than the ones used in this thesis might be validated for their efficiency in rewriting epigenetic marks. Using the review in **chapter 2**, such domains might be fused to ZFs in order to upregulate gene expression. Successful targeted induction of gene expression has been achieved with other histone acetyltransferases than p300, histone methyltransferases acting on H3K79 or H3K4 and histone demethylases acting on H3K9 methylation.

Repression of gene expression through Epigenetic Editing has already been achieved. Furthermore, successful upregulation of gene expression has been achieved with locus-specific targeting of epigenetic enzymes and by using ATFs. Thus, combining these two approaches in Epigenetic Editing is likely to eventually lead to realization of gene-specific rewriting of epigenetic marks in order to induce expression of epigenetically silenced genes.

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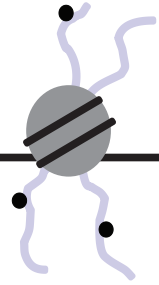
Appendix 1



Nederlandse samenvatting

INLEIDING

Het menselijk lichaam bestaat uit ongeveer 100 biljoen cellen. Het begin van al deze cellen ligt bij de bevruchting van een eicel door een zaadcel. Omdat alle cellen in het lichaam van die ene bevruchte eicel afstammen bevatten ze dezelfde genetische informatie, die bij elke celdeling overgenomen wordt. Ondanks die zelfde genetische informatie, verschillen de cellen in het menselijk lichaam behoorlijk van elkaar. Er zijn bijvoorbeeld spiercellen, levercellen, huidcellen, en nog veel meer verschillende typen cellen. Eén oorzaak van deze verschillen is het verschil in genexpressie. Het DNA dat in elke cel gelijk is, bevat ongeveer 25 duizend genen. Elk van deze genen kan tot expressie koment of niet (aan of uit staan). Daarnaast zijn er nog tussenvarianten waarin een gen zwak tot expressie komt of juist sterk. Dit bepaalt hoeveel van de eiwitten waar het gen voor codeert geproduceerd wordt. Deze eiwitten hebben allemaal hun eigen belangrijke rol in de cel. Afhankelijk van het soort cel (spier/lever/huid enzovoort) zijn bepaalde eiwitten nodig of juist niet nodig. Dus het type cel wordt bepaald door welke genen tot expressie komen en dus eiwitten produceren.

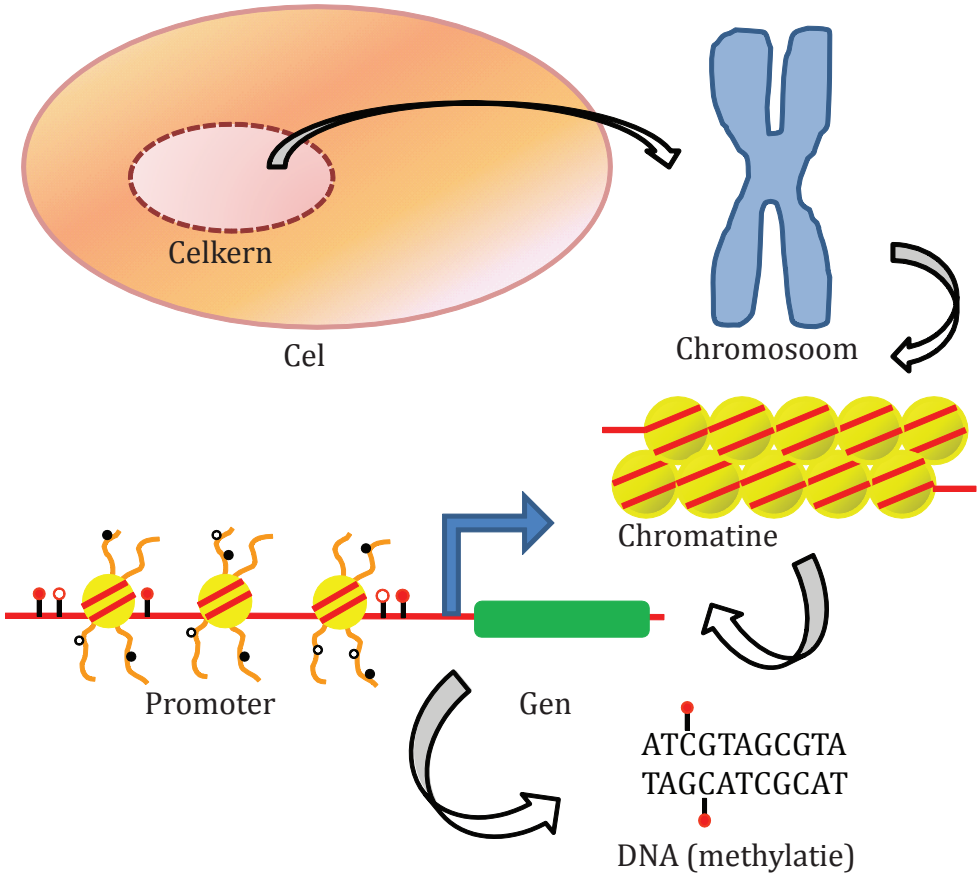
Epigenetica

Een van de mechanismen waarmee een cel de expressie van genen (en dus de eiwitproductie) reguleert is epigenetica. Dit zijn overerfbare en reversibele veranderingen in genexpressie die niet veroorzaakt worden door een verandering in DNA volgorde (genetische informatie). Het DNA van één menselijke cel is ongeveer 2 meter lang. Dit DNA moet passen in de celkern die gemiddeld slechts een doorsnede van 6 micrometer heeft. Dit is te vergelijken met een stuk draad van ongeveer 40 kilometer lang dat in een tennisbal moet passen. Het is dus voor te stellen dat het DNA ingenieus opgevouwen moet worden (gecondenseerd) om in een celkern te passen (Fig. 1). De mate van condensatie van het DNA van een gen bepaalt hoe sterk genen tot expressie komen.

Histon modificaties

De mate van condensatie hangt af van de nucleosoomdichtheid. Een nucleosoom is een cluster van 8 histonen waar DNA omheen gewikkeld is. Elk van de 8 histonen heeft een staart die uit de cluster steekt. Deze staart bestaat uit een reeks aminozuren, waarvan sommigen een beetje veranderd kunnen worden (histonmodificaties). Er bestaan een heleboel verschillende soorten histonmodificaties, die zorgen voor de compactheid waarmee DNA wordt opgevouwen en een hoge of lage nucleosoomdichtheid veroorzaken. Om ervoor te zorgen dat de genen eiwitten kunnen produceren moet het DNA lokaal niet te compact zijn en dus niet teveel nucleosomen bevatten. Er moeten namelijk eiwitten bij het gen kunnen komen om de expressie van het gen te induceren.

Specifiek is van belang hoe de situatie is in de buurt van de promoter van het gen. Dit is een stuk DNA die de aandrijving van expressie van een gen reguleert.



Figuur 1. Epigenetica

Dit is een schematische weergave van de organisatie van DNA in de celkern. In de kern van een mensenlijke cel zitten 23 chromosomen, waarin het DNA opgeslagen ligt. Dit DNA is verpakt in chromatine, een combinatie van DNA en histonen. Een chromsoom bestaat uit meerdere genen. Een gen bestaat heeft een promoter die de expressie van het gen reguleert. Epigenetische marks als histone modificaties en DNA methylatie beïnvloeden de expressie van een gen en daarmee de eiwitproductie zonder de DNA volgorde te veranderen. De gele bolletjes zijn histonen, de rode lijnen representeren DNA. De zwarte stokjes met bolletjes stellen CpGs voor die gemethyleerd (dicht rood bolletje) of ongemethyleerd (open rood bolletje) zijn. De oranje staarten die uit de histonen steken kunnen ook gemodificeerd worden; open zwarte bolletjes representeren genexpressie activerende modificaties, dichte zwarte bolletjes representeren genexpressie remmende modificaties.

DNA methylatie

Naast de histonmodificaties kan ook DNA methylatie zorgen voor een verandering van eiwitproductie zonder de DNA volgorde te veranderen. DNA bestaat in principe uit 4 bouwstenen; adenine, thymine, cytosine en guanine. Adenine en thymine vormen een basepaar in het dubbelstrengs DNA en cytosine en guanine ook. In welke volgorde deze

baseparen naast elkaar zitten is de genetische informatie die overerft van generatie op generatie. Foutjes in de volgorde of de paring van basen (mutaties) die tijdens het leven kunnen optreden kunnen allerlei ziektes veroorzaken. Interessant genoeg kunnen cytosines daarnaast ook met behulp van celbiologische mechanismen veranderd worden. Als een cytosine niet alleen tegenover maar ook náást een guanine zit, in de volgorde CG, dan kan de cytosine base gemethyleerd worden (Fig. 1). Dit betekent dat er een methylgroep aan de cytosine verbonden wordt die er normaal niet zit. Op verschillende manieren kan deze methylatie er voor zorgen dat een bepaald gen niet langer zorgt voor eiwitproductie. Dit kan soms erg nuttig zijn en er samen met bepaalde histonmodificatie voor zorgen dat niet alle genen aanstaan in alle cellen.

Epigenetica in gezondheid en ziekte

In het algemeen zijn epigenetische veranderingen zoals histonmodificaties en DNA methylatie van belang voor het goed functioneren van het organisme in het geheel en de cel in het bijzonder. Net als in de genetische informatie die opgeslagen ligt in de DNA volgorde kunnen er echter ook foutjes optreden in de epigenetische informatie. Deze onbedoelde veranderingen in epigenetische marks kunnen allerlei ziektes veroorzaken, waaronder ook kanker. Dit komt doordat bepaalde belangrijke eiwitten niet meer geproduceerd worden of doordat eiwitten die voor de cel niet goed zijn juist wel, of méér, geproduceerd worden.

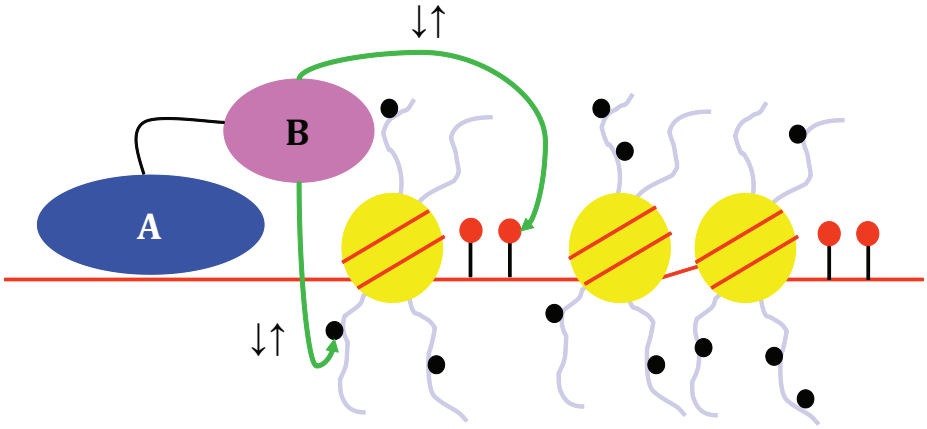
DOEL VAN HET ONDERZOEK

In dit proefschrift staat beschreven hoe we hebben geprobeerd om deze epigenetische fouten te repareren. Het ultieme doel van het onderzoek dat beschreven staat in dit proefschrift was om voor één specifiek gen dat in een bepaalde cel per ongeluk uitgeschakeld staat door foute epigenetische informatie te zorgen dat hij weer aan gaat en dus weer het bijbehorend eiwit produceert. Als dit specifieke eiwit erg belangrijk is voor de cel, zou het er zelfs voor kunnen zorgen dat de cel weer normaal wordt en niet langer 'ziek' is. Dit ultieme doel is geprobeerd te behalen via epigenetische editing.

Epigenetische editing

Epigenetische editing wordt bewerkstelligd door een combinatie van twee factoren (Fig. 2). Aan de ene kant wordt gebruik gemaakt van een eiwit dat specifiek de DNA volgorde kan herkennen van het gen van interesse (Fig. 2a). Eén voorbeeld van zo'n eiwit is een zink vinger, die ontworpen kan worden om elk gen van interesse te herkennen. Eén zink vinger kan namelijk drie baseparen herkennen. Zink vingers kunnen aan elkaar vastgemaakt worden en zo kunnen zes zink vingers aan elkaar dus ongeveer achttien baseparen herkennen. Dit is wiskundig gezien een uniek adres in het humane genoom.

Aan zo'n gen-specifiek DNA bindend domein als een zink vinger wordt dan een epigenetisch effector domein gekoppeld (Fig. 2b). Dit is een enzym dat histonmodificaties kan toevoegen of verwijderen of DNA methylatie kan veroorzaken of opheffen. Afhankelijk van hoe de (foute) epigenetische informatie eruit ziet op het gen van interesse kan gekozen worden uit een heel assortiment van epigenetische enzymen.



Figuur 2. Epigenetische Editing
Dit is een schematische weergave van Epigenetische Editing. (A) stelt het gen-specifieke DNA bindende domein voor (bijvoorbeeld een zink vinger). (B) stelt het epigenetische enzym voor. Met de pijltje wordt de actie van het enzym weergegeven; het toevoegen of verwijderen van een epigenetische mark.

In dit proefschrift was het uiteindelijk doel om specifiek genen tot expressie te brengen. Dit doel is in twee vragen op te breken:

1. Hoe kunnen de epigenetische marks overschreven worden, specifiek op het gen van interesse om genexpressie te induceren?
2. Resulteert deze verandering in epigenetische informatie tot een verhoogde expressie van het gen van interesse?

Afhankelijk van de epigenetische informatie die ervoor zorgt dat het gen van interesse uit staat zou DNA demethylatie (ervoor zorgen dat een cytosine zonder methylgroep de plaats van de gemethyleerde cytosine vervangt) of een bepaalde manier van histon modificatie een weg zijn om te zorgen voor activatie van het gen. Een extra uitdaging in het activeren van genexpressie door Epigenetische Editing ligt in het demethyleren van DNA. Hoewel er bewijs is dat DNA gedemethyleerd kan worden is het nog niet duidelijk welk enzym of welke enzymen er voor verantwoordelijk zijn en via welk mechanisme dit gebeurt.

Dat het veranderen van epigenetische marks een haalbare kaart is, is gebleken met bepaalde medicijnen die al in de kliniek gebruikt worden voor bepaalde kankersoorten. Deze medicijnen blokkeren de werking van bepaalde epigenetische enzymen en zorgen er daardoor na celdeling voor dat de epigenetische marks niet overgenomen worden in

de nieuwe cel. Epigenetische editing heeft een aantal voordelen ten opzichte van deze epigenetische medicijnen:

- Waar de epigenetische medicijnen een effect kunnen hebben op alle genen in het genoom, wordt bij epigenetische editing het effect gestuurd naar één bepaald gen van interesse. Op deze manier is de kans op vervelende bijwerkingen sterk gereduceerd.
- Waar het effect van epigenetische enzymen slechts tijdelijk is (omdat het alleen maar de enzymen remt en niet daadwerkelijk zelf zorgt voor een verandering van de epigenetische marks) is het idee dat epigenetische editing blijvende veranderingen in genexpressie teweeg brengt gezien het kenmerk van epigenetische marks dat ze overerfbaar zijn.
- Waar de epigenetische medicijnen die op dit moment in de kliniek gebruikt worden alleen maar kunnen zorgen voor een inductie van genexpressie, kan epigenetische editing ook zorgen voor remming van genexpressie, afhankelijk van het epigenetische enzym dat gebruikt wordt.

Ook andere benaderingen die momenteel onderzocht worden om genexpressie te veranderen hebben nadelen ten opzichte van epigenetische editing. SiRNA is een benadering waarbij de tussenvorm van gen naar eiwit (RNA) afgebroken wordt en op deze manier de productie van een eiwit wordt verminderd. Het nadeel hier is dat er heel veel RNA aanwezig is in een cel en dat het constant geproduceerd wordt. Het siRNA moet dus constant toegediend worden. Bij Epigenetische Editing wordt het gen zelf getarget, waarvan er maar twee kopieën per cel zijn. cDNA is een kopie van een bepaald gen wat ingebracht wordt in een cel om voor een verhoogde eiwitproductie te zorgen. Het nadeel hiervan ten opzichte van epigenetische editing is dat het gereguleerd wordt door artificiele systemen en dus het eiwit niet in de natuurlijke hoeveelheden produceert. Ook zorgt het maar voor de productie van één vorm van een eiwit terwijl er in de natuurlijk situatie vaak meerdere vormen geproduceerd worden.

HOOFDSTUK 2

Na een algemene introductie in hoofdstuk 1 is in hoofdstuk 2 een review te vinden van het werk van waarbij actief epigenetische enzymen naar een specifieke plek zijn gebracht. In de meeste van de beschreven studies is eerst een stukje synthetisch DNA in het genoom gezet waarna gebruik gemaakt is van DNA bindende domeinen waarvan bekend is dat ze dat stukje DNA kunnen herkennen. Deze DNA bindende domeinen zijn echter niet aan te passen om een bepaald gewenst stukje DNA te herkennen, zoals dat wel het geval is voor zink vingers. Interessant genoeg is in veel van de studies beschreven in het review in hoofdstuk 2 een verandering waargenomen in epigenetische marks of in genexpressie bij het targetgen. In enkele gevallen is zowel een verschil in epigenetische

informatie als in genexpressie bewerkstelligd, wat een indicatie is dat de verandering van epigenetische informatie inderdaad een direct gevolg op genexpressie kan hebben aangezien het targeten van inactief gemaakte enzymen niet tot hetzelfde effect leidde. Zowel activatie of verhoogde expressie van genen als uitschakeling of verminderde genexpressie is bewerkstelligd. Hoewel dit dus veelbelovend lijkt, zijn er slechts een paar voorbeelden bekend van gen-specifieke targeting van epigenetische enzymen. Dit is echter wel van belang om genen van interesse te kunnen beïnvloeden en te voorkomen dat de epigenetische marks van andere genen veranderd worden.

HOOFDSTUK 3

In hoofdstuk 3 is een experimentele studie beschreven waarin we hebben geprobeerd om epigenetische informatie en/of genexpressie te veranderen. Hiervoor is in dit hoofstuk een modelsysteem gebruikt dat bestaat uit cellen met een heleboel kopien van een stukje target DNA erin geïntegreerd. Als DNA bindend domein is in deze study een eiwit gebruikt dat aan één zo'n kopie van dat specifieke stukje target DNA kan binden. Vervolgens zijn aan dat DNA bindende domein verschillende eiwitten gekoppeld waarvan beschreven is dat ze een enzymatische werking hebben op histonmodificaties of DNA methylatie. De enzymen gebruikt in dit proefschrift zijn allemaal beschreven in staat te zijn óf activerende epigenetische marks toe te voegen, óf onderdrukkende marks weg te halen. Daarom is het idee dat het effect van deze specifieke epigenetische enzymen uiteindelijk zou kunnen leiden tot activatie van genexpressie. Het gebruik van de vele kopiën van target DNA zou in theorie kunnen leiden tot een versterkt effect omdat er meerdere epigenetische enzymen tegelijk aanwezig kunnen zijn op een klein gebied. In hoofdstuk 3 hebben we inderdaad aanwijzingen gevonden dat bepaalde enzymen in staat zijn om het DNA wat minder gecondenseerd te maken. Dit leidde vooralsnog nog niet tot significante inductie van genexpressie in een hele celpopulatie. Wanneer gekeken werd naar een specifieke cel werden echter wel indicaties gevonden voor inductie van target genexpressie.

HOOFDSTUK 4

In hoofdstuk 3 werd de werkzaamheid van epigenetische enzymen in een artificieel systeem onderzocht. Om daadwerkelijk Epigenetische Editing te bewerkstelligen zullen deze enzymen echter uiteindelijk naar endogene genen getarget moeten worden. Dit betekent dus dat de epigenetische enzymen maar naar één plek worden getarget in plaats van naar veel kopiën van target DNA zoals in hoofdstuk 3.

In hoofdstuk 4 hebben we voor een bepaald gen, EpCAM, bepaald welke epigenetische marks het gen heeft in eierstokkankercellen. Het bleek dat er eierstokkankercellen zijn waarin EpCAM hoog tot expressie komt en andere eierstokkankercellen waarin EpCAM

laag tot expressie komt. Interessant genoeg bleek deze expressie volledig samen te hangen met de hoeveelheid DNA methylatie in het stukje aan het begin van een gen, de promoter. Als deze promoter onbereikbaar is voor activerende eiwitten vanwege bijvoorbeeld DNA methylatie, kan dit ervoor zorgen dat het gen geen eiwit produceert. Naast de DNA methylatie werden ook bepaalde histon modificaties gevonden die geassocieerd worden met actieve genen of passieve genen. Hierin bleek ook het patroon grotendeels overeen te komen met de expressie van het gen. Bovendien konden we aantonen dat met epigenetische medicijnen de genexpressie gemanipuleerd kon worden. EpCAM lijkt dus een erg interessant modelgen om te zien of we verandering van epigenetische informatie op dat specifieke gen kunnen beïnvloeden en of dit een verandering in genexpressie tot gevolg heeft. Dit is verder uitgevoerd in hoofdstuk 6.

HOOFDSTUK 5

ICAM-1 is een ander gen dat epigenetisch gereguleerd is in eierstokkanker. In hoofdstuk 5 hebben we getest of zink vingers het gen nog steeds konden binden als het epigenetisch uitgeschakeld was. Dit hebben we gedaan door eerder beschreven zink vingers die binden aan het ICAM-1 gen te fuseren aan eiwitten die genexpressie kunnen induceren onafhankelijk van de epigenetische informatie op het target gen. Inderdaad konden we zien dat deze fusie-eiwitten van zink vingers en activatie-eiwitten (VP64) in de eierstokkankercellen waar ICAM-1 uitgeschakeld was door epigenetische informatie konden zorgen voor inductie van ICAM-1 genexpressie. Dit gen is dus ook een interessant modelgen, niet alleen omdat het epigenetisch uitgeschakeld is in bepaalde eierstokkanker cellen maar bovendien omdat het gen weer aan kan worden gezet door fusie-eiwitten met zink vingers. Bovendien zagen we dat die reactivatie van ICAM-1 expressie er voor zorgde dat de kankercellen langzamer gingen groeien.

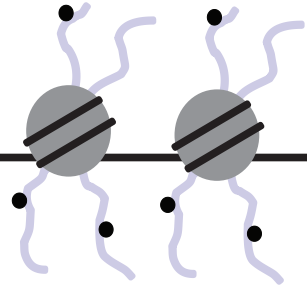
HOOFDSTUK 6

Net als voor ICAM-1 is eerder voor EpCAM beschreven dat na epigenetische silencing van het gen een zink vinger gefuseerd aan VP64 kan zorgen voor inductie van genexpressie. Daarom hebben we in hoofdstuk 6 deze informatie gebruikt om Epigenetische Editing toe te passen. De epigenetische enzymen die getest zijn in hoofdstuk 3 (en andere) werden in hoofdstuk 6 gefuseerd aan de eerder beschreven zink vingers voor EpCAM en ICAM-1. Na behandeling van de eierstokkankercellen met de zink vinger fusie-eiwitten gebruikt in dit hoofdstuk konden geen verschillen gedetecteerd worden in de epigenetische informatie van de beide genen. Ook was er geen verschil in de expressie van de genen te zien. Dit kan het gevolg zijn van vele factoren, waar in de discussie (hoofdstuk 8) op terug gekomen wordt.

CONCLUSIE

Uit het onderzoek wat beschreven is in dit proefschrift valt te concluderen dat er nog veel onderzocht moet worden voordat de activatie van onbedoeld uitgeschakelde genen via Epigenetische Editing een feit is. Er zijn veel factoren die een rol spelen in de expressie van genen en die factoren kunnen elkaar makkelijk en snel beïnvloeden. Het is belangrijk dat gezocht gaat worden naar de meest effective modificatie voor activatie van genexpressie via Epigenetische Editing. Lastig genoeg is de kans groot dat dit niet één modificatie, maar een combinatie van modificaties betreft. Als eenmaal genspecifieke activatie van genexpressie via Epigenetische Editing is bewerkstelligd zijn er nog vele opties om de effectiviteit te vergroten en te optimaliseren. Bijvoorbeeld is het waarschijnlijk nodig om te zorgen voor daadwerkelijke genspecificiteit, zodat niet per ongeluk andere genen ook beïnvloed worden door het getargete epigenetische enzym. Ook moet verzekerd worden dat de enzymen niet al ergens een effect hebben voordat ze bij hun specifieke target aangekomen zijn. Met de vooruitgang in dit onderzoeksgebied (recente publicaties van Epigenetische Editing om genen uit te schakelen en vele andersoortige targeting studies die met epigenetische enzymen genen aan zetten) komt het gebruik van Epigenetische Editing als een gereedschap voor het onderzoeken van het effect van bepaalde epigenetische informatie op specifieke genen van interesse binnen handbereik. Ook de biologische functie van genen (en de eiwitten die ze produceren) kan makkelijker en op een meer natuurlijke manier worden onderzocht door ze via Epigenetische Editing aan of uit te zetten vanaf de natuurlijk promoter dan via de conventionele weg (siRNA en cDNA). Mocht dit alles uiteindelijk succesvol werken en goed geoptimaliseerd zijn, dan zou Epigenetische Editing eventueel zelfs een nieuwe therapie voor kanker en andere epigenetisch gereguleerde ziektes worden.

Appendix 2



Lijst van publicaties en biografie

LIJST VAN PUBLICATIES

Niessen RC, Hofstra RM, Westers H, Ligtenberg MJ, Kooi K, Jager PO, de Groote ML, Dijkhuizen T, Olderode-Berends MJ, Hollema H, Kleibeuker JH, Sijmons RH. Germline hypermethylation of MLH1 and EPCAM deletions are a frequent cause of Lynch syndrome. *Genes Chromosomes Cancer.* 2009 Aug;48(8):737-44.

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van der Gun BT, de Groote ML, Kazemier HG, Arendzen AJ, Terpstra P, Ruiters MH, McLaughlin PM, Rots MG. Transcription factors and molecular epigenetic marks underlying EpCAM overexpression in ovarian cancer. *Br. J. Cancer.* 2011 Jul 12;105(2):312-9.

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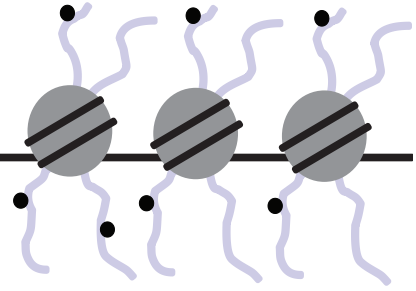
BIOGRAFIE

Marloes de Groote werd geboren op 30 januari 1985 in Groningen. Na het behalen van haar VWO-diploma aan het Augustinuscollege in Groningen, begon zij in 2003 aan de Bachelor-opleiding Life Science & Technology aan de Rijksuniversiteit Groningen. In 2006 vervolgde zij dit met de master Biomedical Sciences aan dezelfde universiteit. Tijdens haar opleiding deed zij verschillende onderzoeksprojecten, bij de afdelingen Straling en Stress celbiologie, Medische Genetica, Ontwikkelingsgenetica en Moleculaire Bacteriologie, van het UMCG en de Rijksuniversiteit Groningen. Na het behalen van haar Master-titel in 2008 begon zij met haar promotieonderzoek bij de afdeling Pathologie en Medische Biologie van het UMCG. Onder leiding van professor Marianne Rots verrichte zij onderzoek naar het gen-specifiek reactiveren van epigenetisch uitgeschakelde genen. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Sinds september 2012 is Marloes werkzaam bij QPS Netherlands B.V. in Groningen, een Contract Research Organisation, alwaar ze Medical Writer/Clinical Research Associate is in het kader van klinische studies.

BIOGRAPHY

Marloes de Groote was born on January 30, 1985 in Groningen, the Netherlands. After obtaining her high school degree at the Augustinuscollege in Groninen, she started in 2003 with the Bachelor study Life Science & Technology at the University of Groningen. In 2006 she started the master Biomedical Sciences at the same university. During her studies she performed several research projects at the departments of Radiation and Stress Cell Biology, Medical Genetics, Developmental Genetics and Molecular Bacteriology of the University Medical Center Groningen (UMCG) and the University of Groningen. After obtaining her Master of Science degree in 2008, she started her PhD project at the department of Pathology and Medical Biology of the UMCG. Under supervision of professor Marianne Rots, she performed research on the gene-specific reactivation of epigenetically silenced genes. The results of this research are presented in this thesis. Since September 2012 Marloes is working at QPS Netherlands B.V. in Groningen, a Contract Research Organisation, where she is Medical Writer/Clinical Research Associate in the context of clinical trials.

Appendix 3



Dankwoord

DANKWOORD

En dan, aan het eind van het promotietraject rest daar nog het dankwoord. Want uiteraard had ook ik, net als alle promovendi die mij voor gingen, mijn proefschrift niet tot een goed einde kunnen brengen zonder de hulp van een heleboel mensen. Ik wil dan ook beginnen met een algemeen woord van dank voor iedereen die een bijdrage heeft geleverd aan mijn proefschrift, op welke manier dan ook.

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